MECHANISTIC STUDIES ON TISSUE TRANSGLUTAMINASE AND ITS ROLES IN CELL GROWTH AND SURVIVAL

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MECHANISTIC STUDIES ON TISSUE TRANSGLUTAMINASE AND ITS ROLES IN CELL GROWTH AND SURVIVAL

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Tissue transglutaminase (tTG) is a dual functional acyl transferase/GTPase that has important roles in many cellular processes, as well as in the development of various disease states. Here we show that tTG expression is frequently up-regulated in high-grade brain tumors or Glioblastoma (GBM), and that tTG is essential for the growth and survival of these highly aggressive cancer cells. We discovered a novel mechanism through which tTG mediates the transformed phenotypes of GBMs, which involves its ability to enhance the signaling activity of the cell surface receptor tyrosine kinase and proto-oncogene, the epidermal growth factor receptor (EGFR). In particular, tTG can associate with and functionally inactivate the E3 ubiquitin ligase c-Cbl, a negative regulator of EGFR, thus delaying its downregulation and extending its signaling half-life. Interestingly, the ability of tTG to influence c-Cbl function is independent of its crosslinking activity, but dependent on a specific conformation that tTG adopts.

X-ray crystallographic studies showed that tTG can exist in a nucleotide-bound, crosslinking inactive closed conformation, or a nucleotide free, crosslinking competent, open conformation. Previous findings from my first study and by others showed that ectopically expressed forms of tTG that adopt a closed conformation in cells, promote cell growth and survival, whereas tTG mutants that adopt an open conformation trigger cell death. Thus, we set out to better understand the molecular mechanisms that regulate the transition of tTG between

these two different conformational states. Two pairs of intramolecular hydrogen bonds formed between the C-terminal end of tTG and its catalytic core domain were identified and shown to play an important role in maintaining tTG in its closed conformation. Disrupting these interactions causes tTG to adopt a constitutively open configuration and trigger cell death. Collectively, these new insights into the regulation and function of tTG have not only led to its identification as an important player in brain tumor progression, but also raise the possibility that developing strategies that cause tTG to adopt an open conformation could be beneficial for the treatment of human cancers.

BIOGRAPHICAL SKETCH

Jingwen was born and raised in Nanchang, Jiangxi, a beautiful city in the southeastern part of China. Even though her parents tried hard to educate her to become an all-around person, Jingwen has always shown stronger interests in Math and Sciences, but struggled in her Chinese and Literature classes. She knew becoming a scientist would be a more feasible goal than becoming a lawyer. After she graduated from high school, Jingwen decided to study Biology at Wuhan University, which had one of the top Biology programs in the country. During her undergraduate years in Wuhan, Jingwen enjoyed the long study hours, hot summer days, and occasional choir practices. She was also exposed to scientific research for the first time when she volunteered to work as an undergraduate research assistant there. Under the guidance of Professor Ying Zhu and Jianguo Wu, Jingwen investigated the cellular inflammatory responses triggered by HIV and SARS virus infections, and developed a strong interest in disease related scientific research. After getting her Bachelor's degree, Jingwen decided to pursue a Ph.D. degree in the United States to further her interests in sciences. Fortunately, she was admitted to the field of Pharmacology at Cornell University and joined Dr. Richard Cerione's laboratory for her graduate study. In the Cerione lab, Jingwen focused on the study of signaling pathways that promote aberrant cell growth and oncogenic transformation, and particularly how the dual function enzyme tissue transglutaminase contributes to brain tumor development. During her six years at Cornell, Jingwen had a great experience in the Cerione lab, and enjoyed the quiet and fun life in Ithaca. After getting her Ph.D., Jingwen plans to get postdoc training and continue to develop as a cancer biologist.

To my family, my mentors, and my friends.

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AD: Alzheimer's disease

AR: amphiregulin

Bax: Bcl-2–associated X protein

Bcl-2: B-cell lymphoma 2

BFA: brefeldin A

BH3: Bcl-2 homology (BH) domain 3

BPA: biotinylated pentylamine

BTC: betacellulin

Cat-1/GIT1: Cool associated tyrosine phosphorylated 1/G protein-coupled receptor kinase interacting ArfGAP 1

c-Cbl: Casitas B-lineage Lymphoma proto-oncogene

c-Met: hepatocyte growth factor

CCPs: clathrin coated pits

Cool-1: cloned-out of library 1

DAG: diacylglycerol

ECM: extracellular matrix

EGF: epidermal growth factor

EGFR: epidermal growth factor receptor

EGFRvIII: EGFR variant type III

EMT: epithelial to mesenchymal transition

EphrinA5: Ephrin receptor A5

EPR: epiregulin

ER: endoplasmic reticulum

ERK: extracellular signal-regulated kinases

FAK: focal adhesion kinase

FRMD4A: FERM domain containing 4A

G-proteins: guanine nucleotide-binding proteins

Gab1: Grb2-associated-binding protein 1

GBM: glioblastoma multiforme

GPCR: G-protein coupled receptor

Grb2: growth factor receptor-bound protein 2

HB-EGF: heparin-binding growth factor

HD: Huntington's disease

HPR: fenretinide/4-hydroxy(phenyl)retinamide/4-HPR

HSP70: heat shock protein 70

IκBα: nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha

IP3: inositol trisphosphate or inositol 1,4,5-trisphosphate

JAK1: Janus kinase 1

JNK: c-Jun N-terminal kinase

K-19: keratin-19

KO: knockout

LAP: latency-associated peptide

LBTP: latent TGFβ -binding protein

MDC: monodansyl cadaverine

MEK: mitogen-activated protein kinase kinase

MVs: microvesicles

MVBs: multivesicular bodies

NF-1: neurofibromin 1

NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells

NHAs: normal human astrocytes

NLS: nuclear localization sequence

P-loop: phosphate-binding loop

PD: Parkinson's disease

PDGFR: platelet-derived growth factor receptor

PGC-1α: peroxisome proliferator-activated receptor gamma coactivator 1-alpha

PI3K: phosphatidylinositide 3-kinase

PIP2: phosphatidylinositol 4,5-bisphosphate

PKC: protein kinase C

PLC: phospholipase C

PTB: phospho-tyrosine-binding motif

PTEN: phosphatase and tensin homolog

RA: retinoic acid

RAREs: retinoic acid response elements

RARs: retinoic acid receptor

RB: retinoblastoma protein

ROCK: RhoA and Rho-associated protein kinase

SH2: Src homology 2 domain

SHP1: Src homology region 2 domain-containing phosphatase-1

SOS: son of sevenless

STAT: signal transducer and activator of transcription

TAO1: thousand and one amino acid kinases 1

TG: transglutaminase

TGF-α: transforming growth factor alpha

TGF-β: transforming growth factor beta

TNFα: tumor necrosis factor alpha

tTG: tissue transglutaminase

VEGF: vascular endothelial growth factor

CHAPTER 1

Overview

Tissue transglutaminase (tTG) is a multi-functional protein that has pleotropic roles in mammalian cells under physiological settings, as well as has been implicated in the progression of several disease conditions. Although originally identified in 1957 (*1*), it has only been in the last 10 years or so that an increasing number of findings have revealed a previously unappreciated role of tTG in promoting human cancer progression. Increases in the levels of tTG expression have been detected in various types of cancer, including breast, pancreatic and ovarian cancer (*2-6*). In this thesis, I set out to better understand the mechanisms through which tTG contributes to oncogenic transformation.

The purpose of this chapter is to provide an overview of tTG and tTG-mediated cellular processes. I will begin by giving a brief introduction of the transglutaminase family, followed by a more detailed overview of tTG, which is the most well-studied transglutaminase family member as well as the focus of this thesis. The two major activities exhibited by tTG, its enzymatic transamidation/crosslinking activity and its GTP-binding/hydrolysis (GTPase) activity, will be introduced, and several of the mechanisms that regulate tTG activation will be highlighted. Then I will focus on the roles that tTG has in the development of two disease states, neurodegeneration and cancer. In the second part of this chapter, I will introduce the epidermal growth factor receptor (EGFR), a critical driver of human malignancies, especially in brain tumors. This part of the thesis serves to provide the necessary background information that will be needed to understand the findings presented in Chapter 2, where I discovered an interesting and unexpected connection between EGFR signaling, tTG, and human brain tumor progression.

The Transglutaminase Family

Protein glutamine γ–glutamyltransferases or transglutaminases (TGs) are a family of enzymes that catalyze an irreversible post-translational modification of proteins by forming covalent linkages (crosslinks) between two proteins or between a protein and a polyamine (*7-9*). There are a total of nine members in the TG family; TG 1-7, blood clotting factor XIII, and the catalytically inactive protein band 4.2 (Table 1.1) (*7, 10*). While specific members of this family, like TG-1, TG-2, and blood coagulation factor XIII, have been extensively studied and are relatively well characterized in terms of their tissue distribution and function, others (i.e. TG-3, TG-4, TG-7, and band 4.2) are not. Collectively, TGs are known to play important roles in numerous physiological processes, including blood coagulation, cell envelope formation, bone ossification, spermatogenesis, and heart development (*7, 11*). However, deregulation of these enzymes is also associated with a number of human disease states, including autoimmune disorders, cancer, infectious diseases, and neurodegenerative disorders (*7, 12, 13*).

For example, blood clotting factor XIII is a transglutaminase that plays an essential role in the formation of blood clots. Factor XIII is expressed in the plasma of animals, and it circulates throughout the body as a tetrameric proenzyme (factor XIII-A_2B_2) composed of two catalytic A subunits and two non-catalytic carrier B subunits held together by non-covalent bonds (*14*). This proenzyme is converted to a catalytically active enzyme (XIIIa) via thrombin cleavage of the A subunits followed by its Ca^{2+}-dependent dissociation from the regulatory B subunits. Once the A subunit active site is exposed, the activated factor XIIIa functions by forming γ-glutamyl-ε-lysyl amide crosslinks between fibrin molecules to form stable and insoluble blood clots. Mutations in the genes encoding the A subunit or the B subunit of factor

Table 1.1. Mammalian transglutaminases and their major functions.

The nine different members of the transglutaminase family and their tissue distribution, cellular localization and primary functions are listed. {Adapted from (*7,10*)}

Protein name	Gene	Gene map locus	Residues (Mr, kDa)	Tissue expression	Localization	Prevalent function
Factor XII subunit A	*F13A*	6p24-25	732 (83)	Platelets	Cytosolic, extracellular	Blood coagulation
TG1	*TGM1*	14q11.2	817 (90)	Keratinocytes	Membrane, cytosolic	Cell envelope formation
TG2	*TGM2*	20q11-12	687 (87)	Ubiquitous	Cytosolic, nuclear, membrane, extracellular, cell surface	Cell death/survival, matrix stabilization, cell differentiation
TG3	*TGM3*	20q11-12	693 (77)	Squamous epithelium	Cytosolic	Cell envelope formation
TG4	*TGM4*	3q21-22	684 (77)	Prostate	Unknown	Semen coagulation
TG5	*TGM5*	15q15.2	720 (81)	Ubiquitous	Unknown	Epidermal differentiation
TG6	*TGM6*	20q11	706 (79)	Unknown	Unknown	Unknown
TG7	*TGM7*	15q15.2	710 (80)	Ubiquitous	Unknown	Unknown
Band 4.2	*EPB42*	15q15.2	691 (72)	Erythrocytes	Membrane	Hematopoiesis, membrane skeletal component

XIII are often causes of a class of blood disorders that are characterized by the formation of unstable blood clots and excessive hemorrhage (*15*).

TG-1, also known as keratinocyte transglutaminase, is another member of the transglutaminase family and is involved in the formation of the cornified cell envelope. By crosslinking keratin, incolucrin, lricrin and other structural proteins, TG-1 promotes the formation of the insoluble cornified cell envelope structure, which surrounds skin cells and protects them against water loss and infection (*16*). Inactivating mutations in the gene encoding TG-1 causes the impaired formation and the instability of the cornified cell envelopes, which is the underlying cause of lamellar ichthyosis, a skin disorder that is characterized by scaly and inflamed skin (*17, 18*).

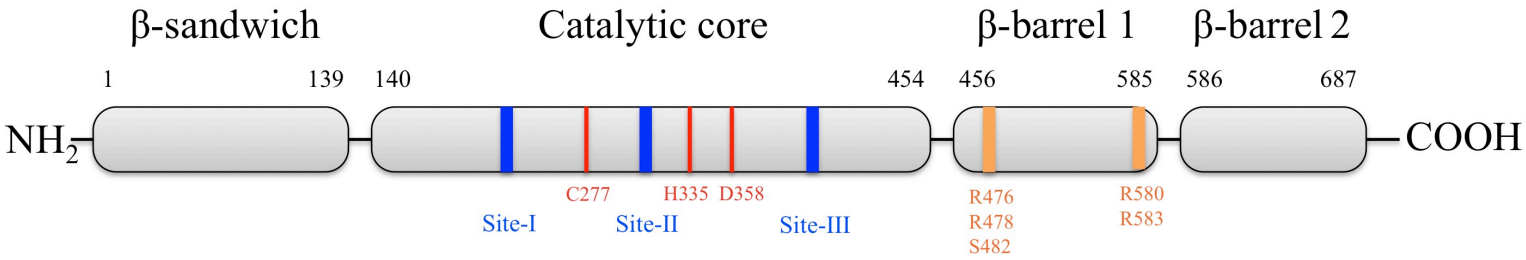
Tissue Transglutaminase: An Important Member of the TG Family

TG-2, which is also referred to as TG-C, TG II, TGase or tissue transglutaminase (tTG) (I will refer to it as tTG throughout the rest of my thesis), is a unique and rather important member of the TG family. It is the most ubiquitously and highly expressed form of all the TG family members, making it the focus of numerous studies. In addition to its conserved transamidation/crosslinking activity, tTG is also capable of carrying out several additional activities, including binding and hydrolyzing GTP (GTPase activity), deaminating proteins, functioning as a serine/threonine protein kinase, and generating protein disulfide bonds (*19*).

Human tTG is a monomeric protein of 687 amino acids composed of four distinct domains; namely the N-terminal β-sandwich (residues 1-139), the catalytic core (residues 140-454), and two C-terminal β-barrel domains (β-barrel 1; residues 479-585, and β-barrel 2; residues 586-687) (Figure 1.1) (*20, 21*). The catalytic core domain of tTG contains the substrate binding

Figure 1.1. Domain organization of tissue transglutaminase.

The four distinct domains of tTG; the N-terminal β-sandwich domain, the catalytic core domain, and the two C-terminal β-barrel domains (β-barrel$_1$ and β-barrel$_2$) are shown. The amino acids that make up each domain are indicated. The catalytic triad, residues involved in binding Ca^{2+}, and residues involved in binding GTP or GDP are listed and highlighted in red, blue and orange, respectively. (*7, 20, 21, 36, 38*)



Catalytic triad: C277
H335
D358

Guanine nucleotide binding sites:
R476, R478, S482
R580, R583

Ca^{2+} binding sites: Asn229/Asp233 (Site-I)
Asp306/Asn310 (Site-II)
Asn398/Glu447 (Site-III)

pocket, as well as the highly conserved catalytic triad that is essential for the transamidation activity of all crosslinking-active TGs. This catalytic triad in tTG, as well as in other TGs, is similar to that seen in cysteine proteinases (i.e. caspases), and includes cysteine 277, histidine 335, and aspartate 358 (*7*). Importantly, mutation of any one of these residues generates a catalytically defective form of the enzyme. For example, the only catalytically inactive member of TG family is the erythrocyte membrane protein band 4.2, which harbors substitutions at all three sites of the catalytic triad (*22*). Moreover, mutating cysteine 277 to a valine in tTG (tTG C277V) completely abolishes its enzymatic activity (*23*).

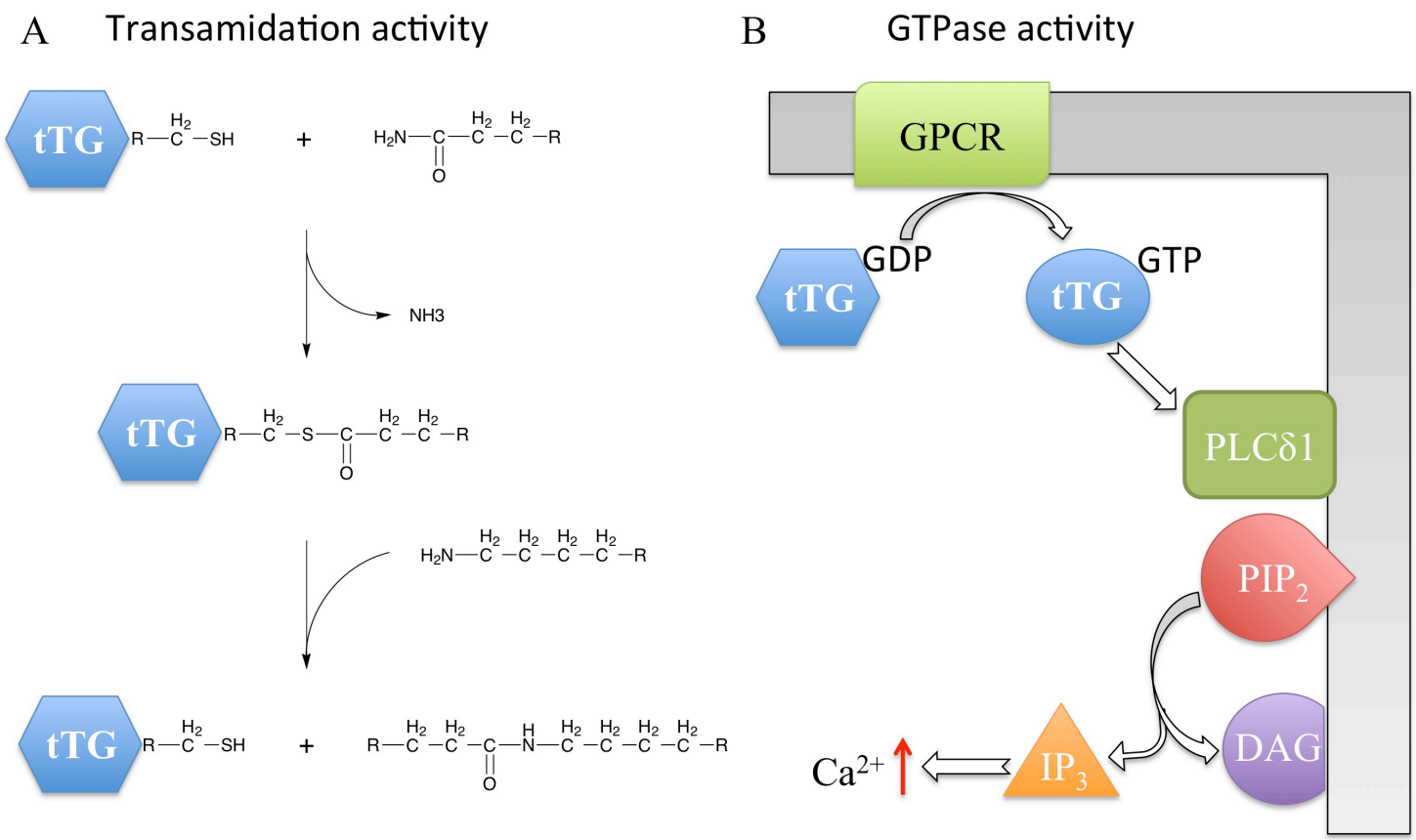
tTG's transamidation activity has been implicated in a wide range of cellular processes, including axonal growth, bone development, wound healing, blood vessel formation (angiogenesis), cellular differentiation, and apoptosis (*24-30*). In the transamidation reaction catalyzed by tTG, the active site cysteine residue reacts with the γ-carboxamide group of an incoming glutamine residue of a protein/peptide substrate to yield a thioacyl-enzyme intermediate and ammonia. The thioacyl-enzyme intermediate then reacts with a nucleophilic primary amine substrate, resulting in the covalent attachment of the amine-containing donor to the glutamine acceptor substrate and regeneration of the cysteine residue at the active site (Figure 1.2A). The end product of the transamidation reaction catalyzed by tTG is the formation of an isopeptide bond between two proteins and the release of ammonia. Importantly, these crosslinks are very stable and resistant to mechanical challenges and proteolytic degradation.

Another activity of tTG is its ability to bind to and hydrolyze GTP, similar to the classical guanine nucleotide-binding proteins (G-proteins). G-proteins function as molecular switches and transition between a GTP-bound active state and a GDP-bound inactive state (*31*). The ability of G-proteins to go between these two states is critical for transducing signals that regulate a myriad

Figure 1.2. The transamidation and GTPase activities of tTG.

(A) tTG-catalyzed transamidation reaction. In the first step, the active site cysteine of tTG reacts with the peptidyl glutamine substrate to form a thioester intermediate and a molecule of ammonia. The thioester intermediate then undergoes a nucleophilic attack by a second protein with a free ε-amino lysine group or by a polyamine. A stable isopeptide bond is then generated between the two substrates and the active site cysteine from the enzyme is recovered and released.

(B) Stimulation of transmembrane GPCRs induces the exchange of GDP for GTP on tTG and activates tTG's signaling activity. The GTP-bound form of tTG then activates PLCδ1, which subsequently catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) to diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3), causing an increase in the intracellular Ca^{2+} concentration.


A
Transamidation activity
tTG
NH3
B
GTPase activity
GPCR
GDP
GTP
tTG
tTG
PLCδ1
PIP2
DAG
IP3
Ca2+

of cellular events. tTG was initially identified and characterized as an atypical, high-molecular weight G-protein, know as Ghα, which mediates signaling from the α_{1B}-adrenergic receptor expressed on the cell surface to phospholipase C (PLCδ-1) (Figure 1.2B) (*32*). Subsequently, the GTP-bound form of tTG was shown to also mediate the activation of Ca^{2+}-activated K^+ channels in vascular smooth muscle cells (*33*).

However, the GTP-binding site of tTG is unique amongst the GTP-binding superfamily of proteins, sharing no homology with the hetero-trimeric G-proteins or the small G-proteins. Specifically, in both the large and small GTPases, there is a highly conserved phosphate-binding loop (P-loop) that is essential for binding to the phosphate moiety of the guanine nucleotide (*34*). The crystal structure of the nucleotide-binding pocket of the small GTPase Cdc42 bound to a GTP analog (GMP-PCP), with its P-loop highlighted, is shown as an example in Figure 1.3A (*35*). In contrast, tTG has a unique guanine nucleotide binding domain composed of a stretch of positively charged residues, including Arg 476, Arg 478 and Arg 580, which contribute to GTP or GDP binding (Figure 1.1 and 1.3B). One of the most important of these residues is Arg580, which forms three hydrogen bonds, one with the guanine ring, one with the α-phosphate, and the last with the β-phosphate of the GDP molecule (Figure 1.3B, right panel). Mutation of this arginine to a lysine residue (tTG R580K) results in a form of tTG that is no longer able to bind GTP (*36*).

Regulation of tTG Activities

tTG's enzymatic transamidation activity is highly regulated and several co-factors have been identified that influence its activity, including Ca^{2+} and GTP or GDP (*20, 36-40*). *In vitro* transamidation assays require millimolar concentrations of Ca^{2+} to fully activate tTG's

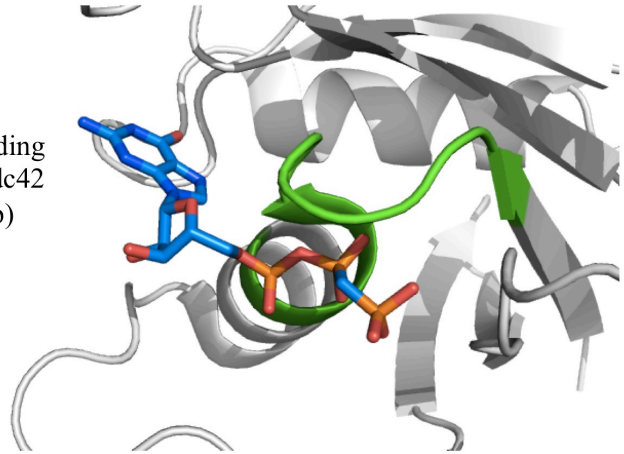
Figure 1.3. Comparison of the nucleotide binding pocket between Cdc42 and tTG.

(A) Close-up view of the nucleotide binding site from the x-ray crystal structure of Cdc42 bound to a GTP analog (GMP-PCP) (PDB: 2QRZ). GMP-PCP is shown in sticks, and the residues coordinating nucleotide binding (the P-loop) are highlighted in green. (*35*)

(B) Close-up view of the nucleotide binding site from the x-ray crystal structure of tTG bound to GDP (PDB: 1KV3). In this structure, GDP is shown in sticks, and the residues coordinating GDP binding are highlighted in green. Interactions between GDP and several key residues in the nucleotide binding pocket of tTG are also highlighted. Polar contacts between GDP and these residues are colored in red. (*21*)

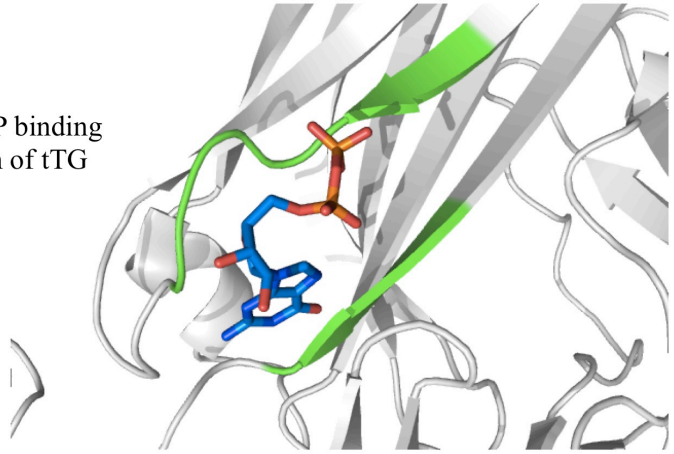
A

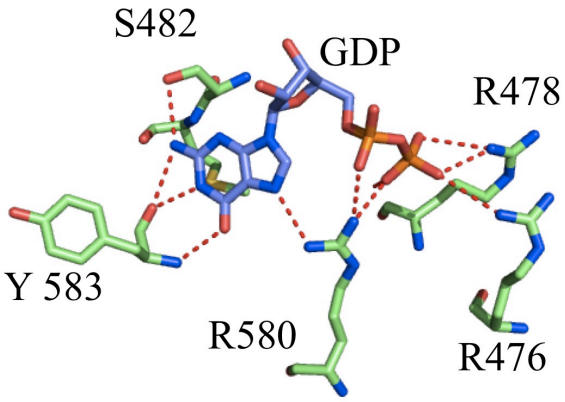
The GTP binding domain of Cdc42 (the P-loop)



B

The GTP binding domain of tTG





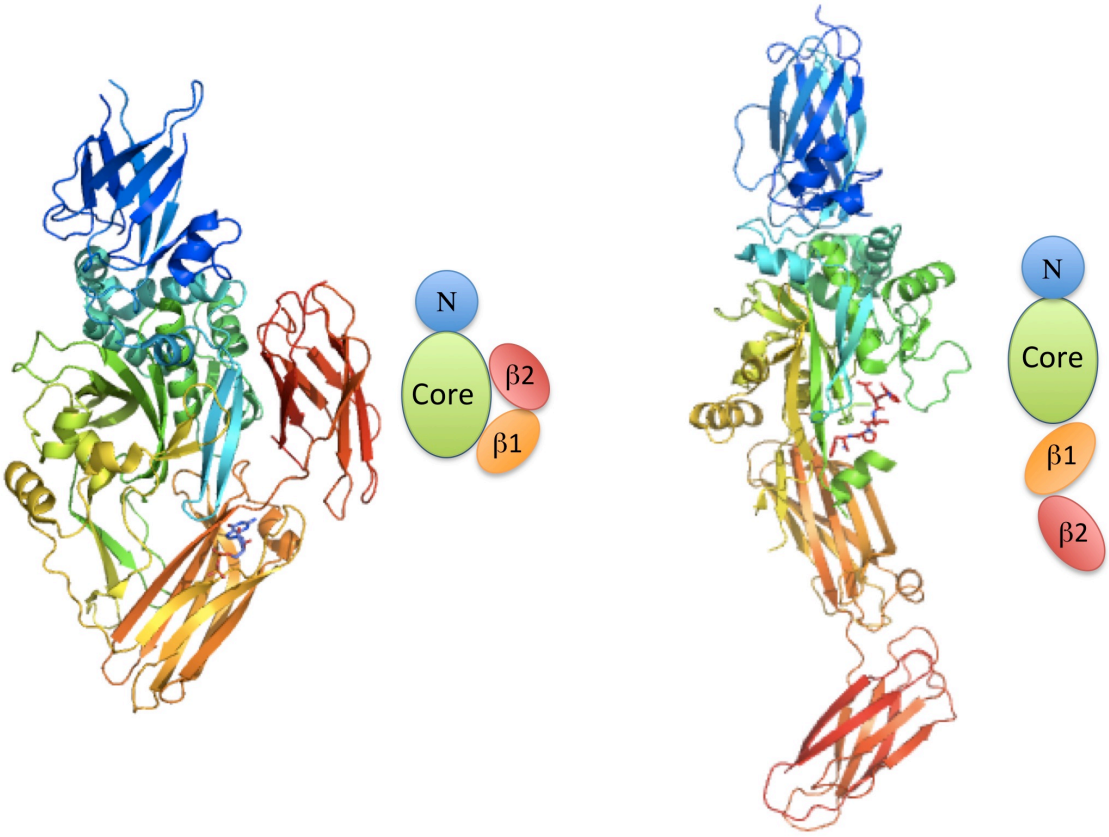
transamidation activity (*37*). Incubation of human recombinant tTG with increasing concentrations of GDP or GTPγS, a non-hydrolyzable form of GTP, showed a dose-dependent inhibition of tTG's transamidation activity, suggesting that the binding of GTP or GDP to tTG negatively regulates its crosslinking activity (*36, 37*). Subsequent x-ray crystallographic studies performed on tTG by our laboratory in collaboration with John Clardy's group showed that the regulation of the transamidation and the GTPase activities of tTG is achieved via allosteric conformational changes that occur within the enzyme following the binding of Ca^{2+} and GTP/GDP (*21*).

The x-ray crystal structure of tTG bound to GDP shows that the guanine nucleotide-binding site of tTG is located in a cleft between the catalytic core domain and the first β-barrel (Figure 1.4, left panel). In this structure, tTG assumes a compact or "closed" conformation where the C-terminal β-barrels (β-barrel 1 and β-barrel 2) fold over and block the access of substrates to its active site located in the catalytic core domain. Because substrates cannot access the active site of the enzyme, this "closed" conformation of tTG is considered to be enzymatically inactive. However, in another x-ray crystal structure solved when tTG was in a nucleotide-free state and bound irreversibly to a peptide that mimics a natural substrate, tTG assumes an extended or "open" conformation where the two β-barrel domains swing away from the core domain and expose the active site for interactions with glutamine containing substrates (Figure 1.4, right panel) (*41*). Thus, this conformational state of tTG is believed to be the enzymatically active state.

Ca^{2+} plays a key role in facilitating tTG's transition from the "closed", inactive conformation to the "open", active conformation (*20, 37*). Although Ca^{2+} was not found in any of the tTG structures, human TG-3 has been co-crystalized with Ca^{2+} (*42*). By comparing

Figure 1.4. Two x-ray Crystal structures of tTG.

The crystal structures of tTG bound to GDP (left) (PDB: 1KV3) and covalently modified with an inflammatory peptide (right) (PDB: 2Q3Z) are shown. GDP and the inflammatory peptide in each structure are shown in sticks and colored in blue and red. The four domains of tTG, including the N-terminal β-sandwich domain (N), the catalytic core domain (Core), the β-barrel 1 domain (β1) and the β-barrel 2 domain (β2), are colored in blue, green, orange and red, respectively. Cartoons representing the organization of the four domains of tTG in each of the structures are also shown. (*21, 41*)


N
Core
β2
β1
N
Core
β1
β2

computational models of tTG with TG-3 bound to Ca^{2+}, our group identified three distinct Ca^{2+}-binding sites in tTG (*38*). These include Site-I (Asn229/Asp233), Site-II (Asp306/Asn310) and Site-III (Asn398/Glu447) (Figure 1.1) (*38*). Studies showed that mutation of any of these sites could inhibit the ability of tTG to crosslink proteins, but they did so to different extents. Mutation of Site-II had the strongest effects on blocking tTG's transamidation activity, while mutations of Site-I and Site-III had more modest effects.

Regulation of tTG Expression

Another important way that tTG activity is regulated is through the regulation of its expression. In certain cell lines (i.e. NIH3T3 mouse fibroblasts, HEK293T human embryonic kidney cells, and SKBR3 human breast cancer cells), tTG is expressed at very low or undetectable levels. However, prolonged exposure of any of these cell lines to a variety of differentiation factors, pro-inflammatory cytokines, growth factors, or hypoxia can up-regulate tTG expression (*26, 43-46*). Moreover, in many other cancer cell lines, especially those that are highly aggressive and metastatic, tTG is constitutively expressed. For example, the human HeLa cervical carcinoma cells, U87 glioblastoma cells, and the MDAMB-231 breast cancer cells all express inordinately high levels of tTG.

Retinoic acid (RA) is one of the best known factors that up-regulate tTG expression (*26, 47, 48*). RA functions as a ligand for the retinoic acid receptors (RARs). Upon binding to RA, RARs become activated and associate with specific sites within the promoter regions of RA-responsive genes known as the retinoic acid response elements (RARE-motifs) and stimulate gene transcription (*49*). In the case of tTG, a RARE-motif was identified in the *TGM2* promoter ~1.7 kb upstream of the transcription start site (*50*). Mutational studies where the RARE motifs

in the *TGM2* promoter were deleted, blocked the ability of RA to induce tTG transcription, highlighting the importance of these elements in the regulation of tTG expression. Importantly, the induction of tTG expression by RA was shown to play an important role in maintaining cell viability during RA-induced cellular differentiation (*26*). This topic will be discussed in more detail later in this chapter.

Activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) signaling pathway was also shown to increase tTG mRNA levels in hepatocytes in response to chemical injury or tumor necrosis factor α (TNFα) stimulation (*51, 52*). NFκB is a widely expressed transcription factor that is typically bound to its negative regulator inhibitor of κB (IκBα) in the cytosol of unstimulated cells (*53*). However, upon stimulation of cells with a variety of growth factors or exposing them to stresses, NFκB rapidly dissociates from IκBα and translocates to the nucleus, where it induces the expression of a specific set of genes that promote cell survival and cell growth. A cognate response element for NFκB was found in the *TGM2* promoter ~1.3 kb upstream of the transcription start site (*48*). Excessive activation of the NFκB pathway may play a particularly important role in inducing tTG expression during inflammation and in many types of cancer cells, which will be further discussed later in this chapter (*54*).

Activation of the epidermal growth factor receptor (EGFR) signaling pathway, which is often de-regulated in human malignancies, is also a known regulator of tTG expression in human breast and brain cancer cells. For example, tTG was identified in a proteomic screen as one of the most highly up-regulated proteins in U87 glioblastoma cells exogenously expressing a constitutively active and highly oncogenic mutant form of the EGFR known as the EGFR variant type III (EGFRvIII) (*55*). Likewise, prolonged exposure of SKBR3 breast cancer cells to

epidermal growth factor (EGF) strongly up-regulated tTG expression and activation from the nearly undetectable levels that are normally seen in these cells (*43*). It was also shown by our laboratory that the combined activation of Ras and Cdc42 downstream of the EGFR, leading to the activation of the phosphatidylinositide 3-kinase (PI3K) and NFκB signaling pathways, was important for the ability of EGF to up-regulate tTG expression (*23*). Increasing the levels of tTG expression and/or activation by EGF in different cancer cell lines was shown to be essential for EGF-mediated cell migration, invasion and anchorage-independent growth, cellular effects which will be described in more detail later in this chapter (*23, 43, 56*). In chapter 2, a novel role for tTG in extending EGFR signaling and promoting brain tumor progression will also be discussed.

The Cellular Localization of tTG

tTG in the Cytosolic Compartment

tTG is expressed in several different compartments within the cell, as well as outside the cell. A majority (~80%) of the tTG is present in the cytosol (*57, 58*). It has been suggested that cytosolic tTG is primarily in the "closed" inactive conformation, due to the low concentration of Ca^{2+} (~100 nM) and the high concentration of GTP (~100 μM) generally found in the cytosol (*59*). It has been proposed that the cytosolic pool of tTG can become enzymatically activated in response to cellular stresses, which frequently results in energy depletion, decreases in GTP levels, and the release of Ca^{2+} from the endoplasmic reticulum (ER) into the cytosol. The net result of these effects triggers the opening of tTG, making its active site accessible to substrates.

tTG in the Nucleus

Subcellular fractionation studies demonstrated that approximately 5-7% of all the tTG expressed in a cell resides in the nucleus (*57*). Several factors, including elevating the levels of intracellular Ca^{2+}, RA treatment, or vascular endothelial growth factor (VEGF) stimulation of endothelial cells, were shown to cause a small amount of tTG to translocate to the nucleus (*57, 60, 61*). Two putative nuclear localization sequences (NLSs) were identified in tTG (amino acids 259-263 and 597-602) (*62*). However, forms of tTG in which the NLSs were mutated still localized to the nucleus like wild type tTG, indicating that the NLSs in tTG are not essential for its trafficking into the nucleus (*63*). Importin-α 3, a nuclear transport protein, was found to associate with tTG both *in vitro* and *in vivo*, raising the possibility that tTG might bind Importin-α 3 as a means to translocate to the nucleus (*62*).

Even though only a small percentage of the tTG expressed in cells is found in the nucleus (~ 5%), nearly ~15% of tTG substrates identified to date are nuclear proteins, suggesting that this small pool of tTG may play important roles in various cellular processes (*10, 57*). This appears to be the case, as tTG-mediated crosslinking of core histones was shown to promote the condensation of chromatin during apoptosis (*64, 65*). Another example of a nuclear substrate of tTG is the tumor suppressor retinoblastoma (RB) protein, a well-known regulator of cell cycle progression and cellular differentiation (*66*). RB is a target of caspase-mediated degradation in p53-induced apoptosis, and RB-knockout mice are embryonic lethal and exhibit elevated levels of apoptosis in the developing retina and throughout the nervous system (*67, 68*). During RA-induced differentiation of erythroleukemia cells, tTG was shown to protect RB from caspase degradation in a transamidation-dependent fashion (*27*). This finding indicates that tTG may protect cells from undergoing apoptosis by modifying RB during RA-induced cellular

differentiation. More recently, it has also been shown that nuclear localized tTG may play critical roles in the broad transcriptional dysregulation that occurs in the mouse model of Huntington's disease (*63*). In this case, tTG is thought to regulate the expression of genes important in regulating cell metabolism either by directly associating with core histones and inhibiting transcription, or by crosslinking chromatin.

Extracellular tTG

The γ-glutamyl-ε-lysine bonds formed as a result of tTG's transamidation activity have also been detected in the extracellular matrix (ECM), indicating that tTG can be externalized from cells (*24, 69*). Nearly every major ECM protein are known substrates of tTG, including fibrinogen, fibronectin, and collagen (*70*). tTG's localization in the ECM plays important roles in promoting cell adhesion, migration, and ECM organization and turnover, with these effects having important consequences in physiological processes, ranging from normal wound healing to tissue regeneration. For example, tTG-mediated crosslinking of fibronectin to itself was shown to increase the rigidity or stiffness of fibronectin polymers and promote cell attachment (*71*). tTG can similarly crosslink and stiffen fibrinogen and collagen fibers (*72, 73*). The net result of these ECM crosslinking events is amplified integrin receptor clustering and focal adhesion formation on the cells that are attached to the ECM, which leads to elevated integrin signaling and activation of downstream focal adhesion kinase (FAK) and extracellular signal-regulated kinase (ERK) (*73*). Activation of FAK and ERK are known to regulate cell spreading and migration, but they also promote cell growth and survival. Thus, tTG modified ECM can impact a number of cellular processes.

tTG is also a regulator of transforming growth factor β (TGFβ) maturation, which is a key event during ECM remodeling (*74*). TGFβ has been shown to increase the synthesis and secretion of major ECM proteins (i.e. collagen and fibronectin), and inhibit the proteolytic degradation of matrix proteins by reducing the activities of proteases that act on ECM (i.e. collagenase and plasminogen activator) (*75*). Under basal conditions, TGFβ is associated with latency-associated peptide (LAP) and latent TGFβ-binding protein (LBTP) in a complex in the ECM. tTG was shown to covalently crosslink LBTP to ECM proteins such as fibronectin, thus promoting the release of TGFβ into the ECM and leading to its reorganization (*7, 76, 77*).

The findings from several studies have established that, in addition to modifying cellular behavior/outcomes by reshaping the ECM via its enzymatic transamidation activity, tTG also exhibits an important non-enzymatic adaptor/scaffold function in cells by promoting the formation of a complex between transmembrane receptors expressed on the cell surface and the ECM. For example, tTG can associate with the cell adhesion receptors, integrins (especially β1-integrins), and promote cell attachment to the ECM by bridging β1-integrin and fibronectin interactions (*78*). The tTG-β1-integrin-fibronectin complex formation drives integrin clustering and activation of downstream signaling events, including the activation of FAK, c-Src tyrosine kinase, RhoA and Rho-associated protein kinase (ROCK) (*79, 80*). In fibroblasts and vascular smooth muscle cells, tTG has also been shown to enhance the association of integrins and the platelet-derived growth factor receptor (PDGFR), leading to enhanced PDGFR clustering and downstream signaling (*81, 82*). Thus, it is clear that under certain circumstances, tTG can function as a scaffold.

The Mechanism Through Which tTG Is Released From Cells

tTG is constitutively secreted from a wide range of cell types (*79, 80, 83*). However, it contains none of the classical secretory signal sequences, nor does it have any hydrophobic domains that are associated with classically secreted proteins, suggesting that tTG is not secreted from cells via the classical secretory pathways that involve the ER and Golgi apparatus (*7, 79, 84*). Consistent with this idea, treatment of cells with brefeldin A (BFA) or Exo1, inhibitors of classical secretion pathways, did not block the release of tTG from cells (*85*).

The mechanism of tTG externalization from cells remained largely unknown until just recently. Studies by our laboratory have discovered a rather surprising way that tTG is secreted from cells, which involves the generation of plasma membrane-derived microvesicles (MVs) (*85*). MVs are extracellular vesicular structures that are formed and shed directly from the surfaces of cells (*86*). They contain a variety of cargo that is not typically thought to be released from viable (healthy) cells, including cell-surface receptor tyrosine kinases, metabolic enzymes, RNA transcripts, and microRNAs (*85, 87-89*). Once released from a donor cell (the cell that generates the MV), the MVs can then be taken up by recipient cells and impact their behaviors (*86*). The transfer of epigenetic information between two cells via MVs represents a novel form of cell-to-cell communication believed to be utilized by all cell types, and especially by aggressive forms of human cancer cells (*90*). Studies by our group have shown that tTG is a major protein component of cancer cell-derived MVs and that its transfer to normal recipient cells (i.e. NIH3T3 fibroblasts and non-transforming MCF10A mammary epithelial cells) caused them to exhibit a transformed phenotype, including increased cell survival and the induction of anchorage-independent growth (*85*). tTG was shown to both bind to and crosslink fibronectin (making a constitutive fibronectin dimer) on the surfaces of these vesicles, which was essential

for their ability to transform normal fibroblasts and mammary epithelial cells (*85*). These findings highlighted at least one mechanism by which tTG can be externalized from cells, and showed that MV-mediated communication between cells might play an important role in cell transformation and cancer progression.

tTG in the Mitochondria

Emerging evidence suggests that tTG might also be expressed in the mitochondria. While tTG does not contain the classical N-terminal mitochondrial targeting sequence found in many proteins that reside in the mitochondria, it has been shown by immunofluorescent microscopy and cell fractionation studies to localize to the mitochondria in various cell types (*91, 92*). Further evidence that tTG associates with the mitochondria comes from studies using tTG knock-out (KO) mice. tTG KO mice are viable, grow to normal size, and show no reproductive defects (*93, 94*). However, these animals have impaired ATP production, particularly after being challenged with exercise (*95*). Moreover, while under normal cellular conditions, tTG activity is tightly regulated, its expression and activation are often increased in several disease conditions that are characterized by mitochondrial dysfunction, including cardiovascular ischemia and neurodegenerative disorders (*96, 97*). The role of tTG in the pathogenesis of several forms of neurodegenerative disorders will be further discussed in the next section.

An eight amino acid stretch in tTG (amino acids 204-211) that shares 70% identity with the Bcl-homology 3 (BH3) domain of B-cell lymphoma-2 (Bcl-2) was recently identified (*92*). Since Bcl-2 is a protein that localizes to the mitochondria and regulates the apoptotic responses in cells, the possibility that tTG may similarly be localized to the mitochondria via this domain and involved in apoptosis was proposed. In line with this idea, tTG was shown to interact with

the pro-apoptotic protein Bcl-2 associated X protein (Bax), but not with the anti-apoptotic protein Bcl-2 (*92*). Bax also serves as a major substrate for tTG-mediated crosslinking in the mitochondria. tTG-induced hyperpolarization of the mitochondrial membrane sensitizes cells to programmed cell death (*91*). Over-expression of tTG in neural cells resulted in a much more rapid induction of cell death that was accompanied by morphological changes to the mitochondria, which included clustering of the mitochondria, reduced cristae size, and an extremely electron-dense matrix (*91*). Collectively, these findings point to a possible connection that may exist between tTG localized to the mitochondria and cellular metabolism and the regulation of cell death.

tTG and Neurodegenerative Diseases

Neurodegenerative disorders include a number of chronic conditions that are characterized by progressive loss of neurons that ultimately results in the death of the patient (*98*). A subset of these disorders are characterized by the aggregation of disease-specific pathogenic proteins, including Parkinson's disease (PD), which is characterized by α-synuclein aggregation and formation of Lewy bodies, Alzheimer's disease (AD), which is characterized by β amyloid and tau aggregation, and Huntington's disease (HD), which is characterized by mutant huntingtin protein aggregation (*99*). Protein aggregate (aggresome) formation in cells is generally believed to function as a protective mechanism to deal with a large amount of misfolded or damaged proteins that have failed to be eliminated by the ubiquitin proteasome system and have accumulated in the cell (*100*). Thus, by forming protein aggregates, the cytotoxic effects of these misfolded proteins are minimized. However, once the aggregates reach

a certain size, they begin to interfere with essential cellular processes and cause neuronal cell death (*101*).

Elevated expression and activation of tTG are frequently observed in HD, AD, and PD patients (*99*). Depletion of energy and increases in the cytosolic Ca^{2+} levels in these diseased cells are believed to induce tTG to adopt an "open", crosslinking competent state (*102*). The covalent crosslinking of pathogenic proteins by tTG into large proteinaceous aggregates are a recurring theme in PD, AD, and HD, and the use of tTG expression and activation have been proposed as a diagnostic marker for these diseases (*98*).

Among the different neurodegenerative disorders that tTG has been associated with, it has been most heavily studied in HD. The hungtingtin (htt) protein normally contains a polyglutamine tract that ranges from 6-35 glutamine residues (*103*). However, when the polyglutamine repeat is expanded by just one glutamine residue (36 glutamines), htt becomes pathogenic. This number of repeats can be as large as 250 glutamine residues in a row, and the length of the glutamine tract directly correlates to the severity of the disease symptoms (*104-106*). Interestingly, tTG can not crosslink a htt protein containing a normal number of glutamines (from 6-35), however, htt proteins of pathological length can be crosslinked by tTG (*103, 107, 108*). Moreover, tTG activity is increased in HD brain tissue, and it colocalizes with htt aggregates in the nucleus, suggesting a direct involvement of tTG in htt aggregate formation (*109, 110*). In mouse models of HD, treatment with a tTG inhibitor, cystamine, reduces htt aggregate formation in the cortex and striatum, and extends the life of these animals (*111, 112*). Crossing the tTG knock-out mouse strain with the HD mouse model resulted in a mouse line that exhibits improved motor performance, prolonged survival and decreased cell death compared to the control HD mice (*113*). More recently, tTG has been implicated in the broad transcriptional

dysregulation in the mouse model of HD, including repression of genes that control mitochondrial metabolic functions, such as cytochrome c and peroxisome proliferator-activated receptor-γ coactivator (PGC)-1α, indicating that tTG may contribute to several aspects of HD development (*63*).

Alzheimer's disease (AD) is the most common neurodegenerative disorder and is another disease in which tTG has been implicated (*96*). β amyloid and tau are the two proteins that have been most often linked to the development of AD. Evidence from *in vitro* and cell culture based studies have shown that tTG can crosslink both tau and β amyloid (*96*). Elevated tTG expression combined with its co-localization with tau and β amyloid aggregates in AD brains further suggest a role for tTG in the development/progression of AD (*114*). Moreover, a splice variant of tTG, called tTG-Short (tTG-S), is commonly found in AD brains, while being virtually undetectable in control normal brains (*115-117*). tTG-S lacks the C-terminal 138 amino acids that correspond to the auto-inhibitory C-terminal domain, and it exhibits greatly reduced transamidation activity and GTP binding activity (*115*). Studies from our group showed that this truncated form of tTG exerts diametrically opposite effects on cell viability compared with full-length tTG, as full-length tTG confers a strong survival advantage to cells whereas tTG-S is apoptotic (*115*). Several ongoing studies are focused on this isoform of tTG in the pathology of AD.

tTG in Cancer Progression

As little as a decade ago, tTG was generally believed to promote cellular differentiation or to induce apoptosis. However, a growing body of evidence suggests a very different role for tTG in human cancers. For example, tTG is over-expressed in various forms of human cancer, where it participates in an array of cellular processes/events that are intimately linked to cancer

progression, including promoting cell growth and survival, as well as driving cell migration and invasion.

tTG in Cell Migration and Invasion

A number of studies have demonstrated a role for tTG in promoting cell migration. The ability of tTG to promote cell attachment and movement depends in part upon its capacity to interact with integrin and fibronectin. The formation of tTG-integrin-fibronectin complexes, where tTG works to bring integrin and fibronectin together more efficiently, stimulates the activity of signaling proteins that are intimately linked to cell migration, including FAK, the MAP kinase ERK, and the small GTPase RhoA (*78-80*). tTG has also been shown to crosslink ECM proteins (i.e. fibronectin and collagen), increasing the rigidity of the ECM, which promotes integrin clustering and activation, and stimulates cell migration (*71-73*). tTG can also potentiate the ability of growth factors to activate signaling events. It does so by interacting with growth factor receptors such as the PDGFR, and mediating their association with integrins to enhance the efficiency of PDGF-induced signaling events (*81, 82*).

Our laboratory identified tTG as an important mediator of EGF-stimulated HeLa cervical carcinoma cell migration and invasion (*56*). EGF stimulates the activation of tTG in HeLa cells without having a significant effect on its expression. Blocking the activity of tTG in HeLa cells by exposing them to the tTG inhibitor monodansyl cadaverine (MDC), or knocking-down tTG expression by siRNA, potently inhibited EGF-stimulated cell migration. This initial study was then followed-up by another study that showed EGF stimulation caused a rapid accumulation of tTG to the leading edges of cells (*118*). Leading edges are plasma membrane protrusions that extend from the cell body in the direction of movement, providing the necessary adhesion that a

cell needs to move forward (*119*). It was then shown that the redistribution of tTG to the leading edges depends on the ability of tTG to interact with the chaperone heat shock protein 70 (Hsp70). Blocking Hsp70 activity using the specific inhibitors myricetin or VER 155008 prevented tTG's localization to the leading edge and blocked cell migration.

In addition to being important in cell migration, tTG is also required for the invasive activity exhibited by some highly aggressive human cancer cells. The first indication that this might be the case came from a study in which tTG was identified in a proteomic screen as one of only eleven proteins that were consistently up-regulated in metastatic human lung and breast tumors (*120, 121*). Moreover, work by our laboratory showed that tTG was preferentially over-expressed in primary breast tumors and breast cancer cell lines that are highly invasive and metastatic (i.e. the MDA-MB-231 human breast cancer cell line) (*43*). The ectopic expression of tTG in MCF10A, a non-transformed mammary epithelial cell line, was shown to be sufficient to induce the epithelial to mesenchymal transition (EMT), a process in which epithelial cells lose their typical cell polarity and cell-cell adhesion properties and acquire a more mesenchymal-like migratory and invasive phenotype (*122, 123*). tTG expression in these normal mammary epithelial cells leads to the signature loss of epithelial-specific markers (i.e. E-cadherin and β-catenin), and the up-regulation of mesenchymal markers, such as fibronectin, and N-cadherin, as well as the transcriptional repressors Twist1, Zeb1, and Zeb2. The up-regulation of Zeb1 and the switch in the expression from E-cadherin to N-cadherin were shown to result from the tTG-mediated activation of the NFκB and Akt signaling pathways, respectively. The tTG-induced EMT promoted the detachment of cancer cells from the primary tumor and facilitated their migration and metastatic activity.

The Protective Effects of tTG

Apoptosis is a form of programmed cell death that is initiated through the activation of specific intracellular pathways in response to various death stimuli, and it plays critical roles in development and normal tissue homeostasis (*124*). Interestingly, cancer cells often develop ways to limit or circumvent apoptosis, providing them with a selective advantage to grow under stressful conditions or when challenged with chemotherapeutic agents. Based on some early findings showing that tTG expression and activation were up-regulated during apoptosis, combined with the fact that tTG is frequently over-expressed and/or overactivated in neurodegenerative disorders (i.e. AD, HD and PD), it is easy to understand why tTG was initially believed to be involved in promoting cell death (*98, 125*). However, more recent findings have shown that the up-regulation of tTG expression in response to cellular stress is often used to help cells cope with the insults rather than promoting cell death. Moreover, it is now clear that cancer cells often hijack tTG and use it to promote cell survival.

One of the first lines of evidence suggesting that tTG can promote cell survival came from a study in our laboratory that was looking at the effects of retinoic acid (RA) treatment on cells (*26*). We found that the exposure of HL-60 leukemia cells to RA induced tTG expression and activation, as well as caused the cells to differentiate into mature granulocytes. However, when the same cells were treated with RA and the tTG inhibitor, MDC, instead of inducing cellular differentiation, the cells died, suggesting that the RA-induced tTG activity was important for maintaining cell viability during cellular differentiation. This was further supported by the finding that exposing HL60 cells to a synthetically-derived analog of RA that is particularly effective at causing cell death, called fenretinide (HPR), triggered an apoptotic response in these cells. It was later shown, that unlike RA, HPR was unable to induce tTG expression. However,

ectopic expression of tTG in HL-60 cells was sufficient to protect these cells from HPR-induced apoptosis. These findings were some of the first showing that the up-regulation of tTG expression and activation did not always result in apoptosis. Rather, they showed that, at least in some cell contexts, tTG can help to tip the balance toward differentiation rather than cell death.

Over-expression of tTG in human embryonic kidney HEK293T cells was also shown to provide protective effects to these cells in response to treatment of the Ca^{2+} ionophore A23187 (*126*). Increases in the cytosolic Ca^{2+} levels due to Ca^{2+} immobilization by A23187 treatment increased mitochondrial permeability and cytochrome c release to the cytosol. These events then led to the activation of caspases, which execute their apoptotic functions by cleaving proteins that are essential for maintaining cell viability, thus causing cell death (*124*). Stable expression of tTG in HEK293T cells was shown to be able to block A23187-induced apoptosis by preventing mitochondrial membrane depolarization and the release of cytochrome c into the cytosol, thus preventing the subsequent activation of caspases (*126*).

Several types of breast, pancreatic and brain cancer cell lines that are resistant to chemotherapy also tend to have high levels of tTG expression and transamidation activity (*2, 127*). A case in point is the highly aggressive and malignant MDA-MB-231 breast cancer cell line, which exhibits inordinately high levels of tTG expression and activation. Knocking down tTG expression in these cancer cells using siRNA or treating them with the transamidation inhibitor MDC sensitized the cells to the chemotherapeutic agent doxorubicin (*128*). Moreover, the survival signaling triggered by the EGFR was also found to be dependent on tTG in some cancer cell lines. For example, the levels of tTG expression and its transamidation activity are markedly enhanced in the human SKBR3 breast cancer cell line after they have been stimulated with EGF for prolonged (~2 days) period of time (*23, 43*). Inhibiting the transamidation activity

of tTG using MDC abolished the ability of the EGFR to protect these cells from doxorubicin-induced apoptosis, as well as from serum-deprivation-induced apoptosis (*43*). Moreover, ectopic expression of tTG in SKBR3 breast cancer cells fully mimics the effects of EGF treatment and desensitizes them to doxorubicin-induced apoptosis.

tTG and Cell proliferation

Accumulating evidence suggests that tTG strongly promotes the growth of human cancer cells (*129*). One mechanism underlying tTG's ability to promote cell growth involves the activation of the transcription factor NFκB (*130*). Increases in NFκB signaling have emerged as a hallmark of human cancers, and a strong correlation exists between the levels of tTG expression and the amount of NFκB activity detected in aggressive and drug-resistant forms of breast cancer cells, malignant melanoma cells, and pancreatic cancer cells (*127, 130, 131*). As discussed in an earlier section, the ability of NFκB to function as a transcription factor and regulate the expression of genes that promote cell growth and survival is normally kept in check by its retention in the cytosol where it is bound to its negative regulator IκBα (*53*). Exposure of cells to a variety of growth factors and cell stresses results in the rapid phosphorylation and degradation of IκBα, thus freeing NFκB to enter the nucleus and influence gene expression. Importantly, tTG was shown to de-regulate NFκB signaling by binding to and crosslinking IκBα both *in vitro* and *in vivo (130)*. The tTG-mediated modification of IκBα reduced its binding affinity for NFκB, thus resulting in the constitutive activation of NFκB transcriptional activity and promoting cell growth.

tTG is also important for the ability of EGF to stimulate the growth of breast cancer cells (*23*). Exposing the human SKBR3 breast cancer cell line to EGF promoted their growth in

monolayer, as well as their ability to form colonies in soft agar, an *in vitro* measure of tumorigenicity. Looking for proteins that function downstream of the EGFR to mediate these effects led to the identification of tTG as a key player in EGFR signaling. Our laboratory showed that tTG expression was not only up-regulated by EGF stimulation, but that knocking-down tTG expression with siRNAs or treating cells with the inhibitor MDC completely blocked the ability of EGF to promote their growth. Moreover, the ectopic expression of tTG in SKBR3 cells was sufficient to mimic the actions of EGF and promote their anchorage-independent growth. The mechanism through which tTG stimulated the growth of these breast cancer cells was then identified and involves the ability of tTG to form a complex with the intermediate filament protein keratin-19 (K19) and the non-receptor tyrosine kinase Src, which is one of the earliest identified oncogenes that contributes to multiple aspects of malignant transformation (*132*). The tTG-K19-Src complex leads to the activation of Src and enhanced cell growth. Since tTG can impact a number of signaling events that are crucial for cell growth and survival (i.e. activating the signaling activities of NFκB and Src), this may start to provide some explanations for how tTG can contribute to several different aspects of cancer progression.

Several lines of evidences have highlighted an intricate connection that exists between EGFR signaling and tTG, namely that tTG functions as a downstream effector of the EGFR and has important roles in EGF-stimulated cancer cell growth and survival (*23, 43, 56, 118*). In Chapter 2 of this thesis, I will describe another unexpected way that tTG contributes to EGFR signaling, which has important consequences in brain cancer progression. In particular, I have found that tTG can help extend the signaling lifetime of the EGFR in some brain tumors. Given

the importance of tTG in EGFR-driven cancers, I will give a brief overview on EGFR and its roles in brain tumor development in the remaining part of this chapter.

Overview Of the Epidermal Growth Factor Receptor (EGFR)

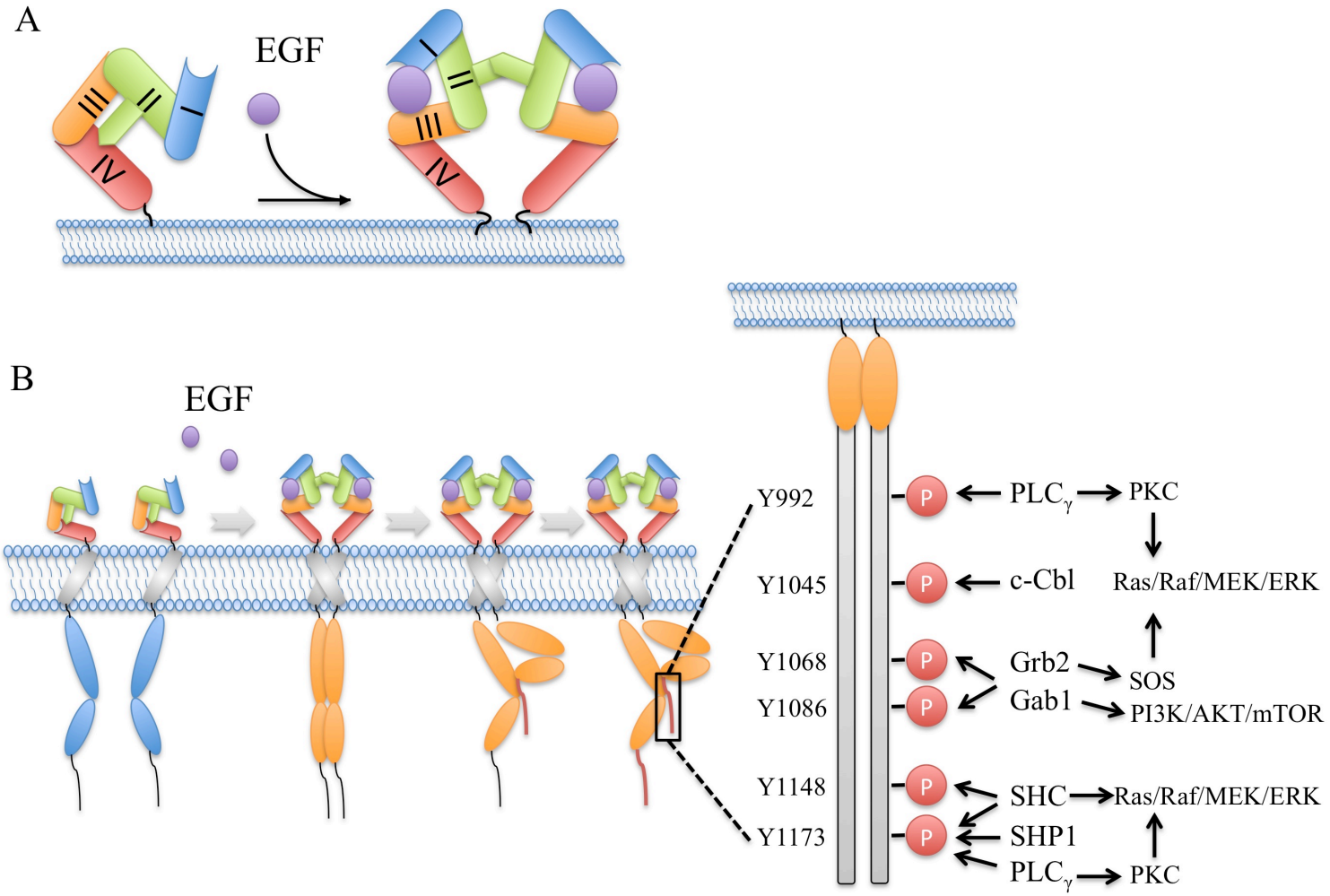
The EGFR is a receptor tyrosine kinase in the ErbB family, which is comprises four closely related members, namely ErbB1/EGFR/HER1, ErbB2/HER2/Neu, ErbB3/HER3 and ErbB4/HER4 (*133*). The gene encoding the EGFR is located on chromosome 7p11-13 and consists of 28 exons (*134*). The EGFR is first synthesized as a 1210-residue polypeptide precursor that contains an N-terminal membrane targeting sequence (*135*). Once trafficked to the cell surface, the membrane targeting sequence of the EGFR is cleaved off, resulting in the mature 1186-residue EGFR protein product that has a molecular mass of ~170 kDa (*136*). Eleven different ligands for the ErbB family have been identified, among which EGF, transforming growth factor α (TGF- α), amphiregulin (AR), heparin-binding growth factor (HB-EGF), epiregulin (EPR), and betacellulin (BTC) can bind to the EGFR and stimulate its tyrosine kinase activity (*137, 138*).

The EGFR is composed of a N-terminal cysteine-rich extracellular ligand-binding domain, a hydrophobic membrane spanning domain, and a C-terminal intracellular tyrosine kinase domain (*139*). The extracellular domain of the EGFR can be further divided into four domains (I, II, III and IV), and can adopt two distinct conformations that regulate its activation (*140, 141*). Domains I and III are each ~160 amino acids in length and are responsible for binding to activating ligands, such as EGF. Domains II and IV are cysteine-rich domains each consisting of ~150 amino acids that are important for promoting the formation of EGFR homodimers or heterodimers with other members of the ErbB family when the receptor is bound

Figure 1.5. The mechanism of EGFR activation.

(A) Schematic representation of the EGF induced dimerization of the extracellular domains of the EGFR. Ligand (EGF) binding induces the transition of the extracellular domain from a tethered auto-inhibited state to an extended conformation. The dimerization arm localized in domain II is exposed in this process, and interacts with the dimerization arm from another EGFR, promoting the dimerization of the ligand-bound EGFRs.

(B) General view of the ligand-induced dimerization and activation of EGFR. EGF binding to the extracellular domain of an inactive EGFR monomer induces its dimerization with another ligand-bound EGFR. The cytoplasmic domains of the dimerized EGFRs are then brought into close proximity to one another, and interact in an asymmetric manner that activates its intrinsic kinase activity. Specifically, the kinase domain of one EGFR phosphorylates the cytoplasmic tail of its dimerization partner. These trans-phosphorylation events create docking sites for downstream signaling molecules. A depiction of the major auto-phosphorylation sites in the cytoplasmic domain of the EGFR along with the signaling pathways associated with these sites are shown in the zoom-in box. {Adapted from (*140, 147*)}


A
EGF
I
II
III
IV
B
EGF
Y992
Y1045
Y1068
Y1086
Y1148
Y1173
P
PLCγ
PKC
c-Cbl
Ras/Raf/MEK/ERK
Grb2
SOS
Gab1
PI3K/AKT/mTOR
SHC
Ras/Raf/MEK/ERK
SHP1
PLCγ
PKC

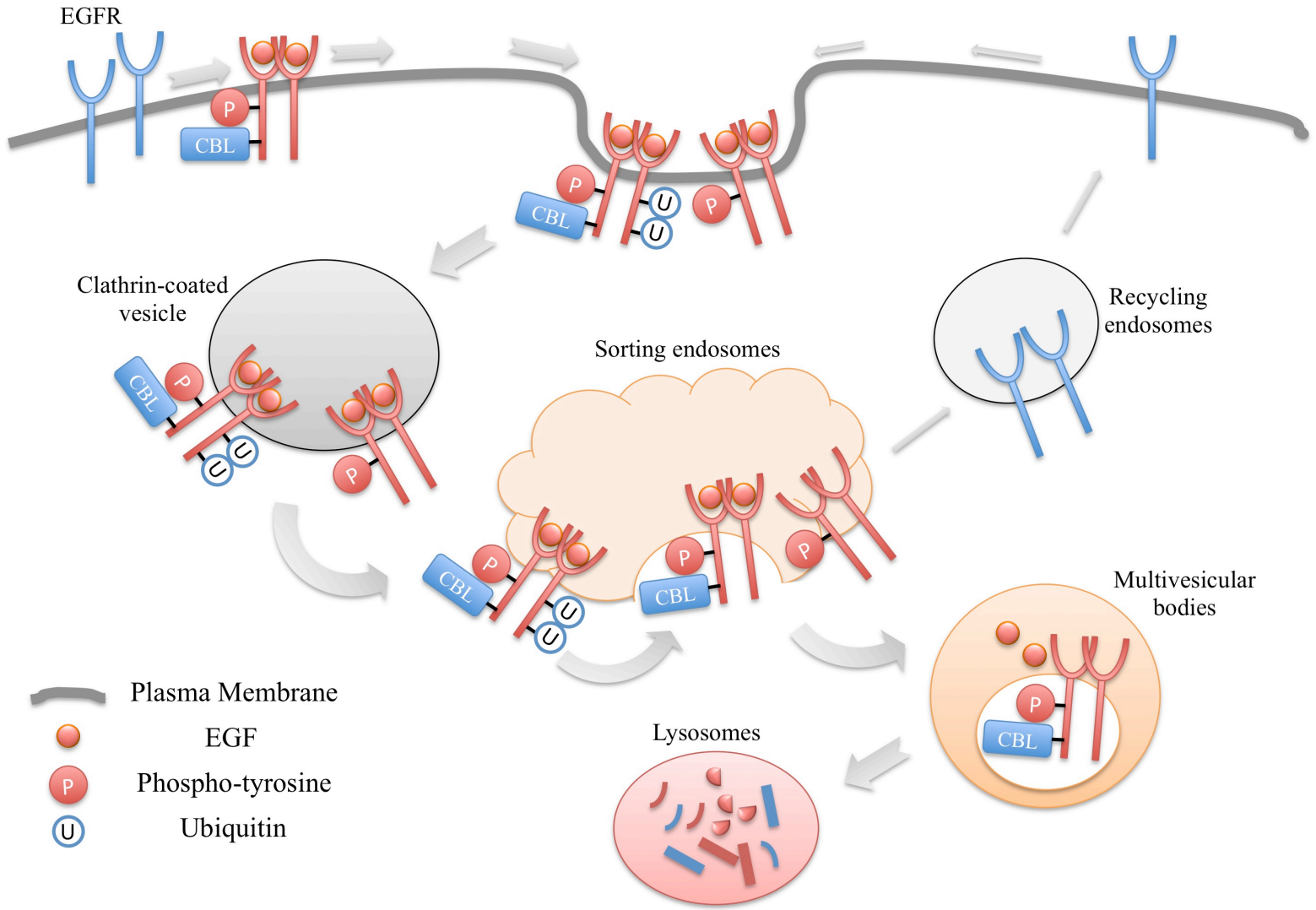
to a ligand (*142*). The conformational changes that occur within the extracellular domain of the EGFR during dimerization is depicted in Figure 1.5 A. Specifically, before ligand binding, the dimerization arm located in domain II is buried by intramolecular interactions with domain IV (*140, 143, 144*). However, upon binding to EGF, domains I and II rotate and transition from the tethered auto-inhibited configuration to an extended configuration, allowing the dimerization arm in domain II to interact with a second ligand-bound receptor (*140, 141*).

Following dimerization of the extracellular domains of the receptors, the intracellular kinase domains dimerize asymmetrically in a head-to-tail orientation. This results in the activation of the intrinsic kinase activity of the EGFR and trans-phosphorylation of specific tyrosine residues within its cytoplasmic tail by the partnering receptor (*145-147*). These residues include Y992, Y1045, Y1068, Y1086, Y1148 and Y1173 (Figure 1.5 B), and their phosphorylation provides docking sites for downstream adaptors and effector proteins. These proteins associate with the EGFR through interactions between Src Homolgy 2 (SH2) domains or phospho-tyrosine-binding (PTB) motifs on the effector proteins with the phospho-tyrosine residues present on the intracellular domain of the EGFR (*148, 149*). After binding to the EGFR, these adaptor and effector proteins stimulate downstream signaling proteins, including ERK (extracellular signal-regulated kinase), PI3K (Phosphoinositide 3-kinase), phospholipase C, protein kinase B (AKT), and STAT (signal transducer and activator of transcription), leading to enhanced cell growth, angiogenesis, migration, and survival (*137, 150*).

Down-regulation of the EGFR signaling activity occurs through a highly regulated, multi-step process that involves EGFR internalization, ubiquitylation, and degradation in the lysosomes (Figure 1.6) (*138*). When the EGFR is activated, a phosphorylation event in its cytoplasmic domain creates docking sites for the ubiquitin ligase c-Cbl (casitas B-lineage

Figure 1.6. Mechanism of endocytic down-regulation of the EGFR.

Ligand (EGF)-induced EGFR dimerization and auto-phosphorylation recruits the E3 ubiquitin ligase c-Cbl to the activated EGFRs. c-Cbl then catalyzes the ubiquitylation of the EGFR, which facilitates the packing of the EGFRs into clathrin-coated endocytic vesicles. The endocytic vesicles first fuse with the sorting endosomes, where the EGFRs are sorted depending on their ubiquitylation status. Ubiquitylated receptors are subsequently routed to the multivesicular body (MVB) and targeted for lysosomal degradation, while non-ubiquitylated receptors are recycled back to the plasma membrane. {Adapted from (*153*)}


EGFR
P
CBL
U
Clathrin-coated vesicle
Recycling endosomes
Sorting endosomes
Multivesicular bodies
Lysosomes
Plasma Membrane
EGF
Phospho-tyrosine
Ubiquitin

lymphoma proto-oncogene). c-Cbl can associate with the EGFR directly by binding to the phosphorylated tyrosine residue 1045, or indirectly through the action of another adaptor protein named Grb2 (growth factor receptor-bound protein 2) (*151, 152*). c-Cbl functions as a ubiquitin ligase and catalyzes the ubiquitylation of the EGFR at several lysine residues within its cytoplasmic tail, which serves as a sorting motif for targeting activated EGFRs for degradation in later steps (*153*). During its internalization, the receptor-ligand complex on the cell surface is first incorporated into clathrin-coated pits (CCPs) that pinch off from the plasma membrane and form the endocytic vesicles. These endocytic vesicles then fuse with the early endosomes and release the receptor-ligand complex into the early endosomes (*154*). After its translocation into the early endosomes, the receptor can undergo either one of two distinct processes depending on its ubiquitylation status (*153*). Those receptors that are not ubiquitylated are recycled back to the cell surface, while the ubiquitylated receptors are taken up into the multivesicular bodies (MVBs) and targeted for lysosomal degradation. Several studies have shown that activated EGFRs continue to recruit and activate intracellular signaling molecules following their internalization and trafficking to the endosomes (*155-157*). Eventually, the acidic environment of the lysosomal lumen causes the ligands to dissociate from the EGFRs. The receptors are then de-phosphorylated, and their signaling activities are terminated (*158*).

The EGFR and Cancer Progression

EGFR and its related family members are the major mediators of a complex signaling network that regulates normal cell growth, differentiation, adhesion, migration, and survival. Studies have shown that EGFR signaling is essential for early embryonic development and in the renewal of adult stem cells in different tissues such as the skin, liver and gut (*159*). EGFR

knock-out mice exhibited severe developmental defects in the epithelial structures of the skin, lung, and the central nervous system (*160, 161*). Collectively, these findings underscore the importance of EGFR signaling in normal development and in tissue maintenance.

However, EGFR-mediated signaling is often de-regulated in human cancers. One of the first pieces of evidence supporting a role for the EGFR in promoting oncogenesis came from the finding that the product of the v-erbB oncogene from avian erythroblastosis virus corresponds to a truncated and constitutively activated form of the EGFR (*162*). The gene encoding the EGFR was later shown to be amplified and/or mutated in primary human brain tumors (*163*). Now it is well appreciated that aberrant EGFR signaling plays a key role in cellular transformation, and that over-expression and/or overactivation of the EGFR is a hallmark of various types of cancers, including breast, colon, pancreas, lung, esophageal, and brain cancers (*133*). Several different mechanisms can give rise to de-regulated EGFR signaling (*164*). The most common one involves amplification of the gene encoding the EGFR. Alternatively, oncogenic EGFR signaling can occur through the acquisition of activating mutations in the EGFR gene. The mechanisms leading to aberrant EGFR signaling activities are perhaps most often seen in a type of highly aggressive brain tumor known as grade IV astrocytoma or glioblastoma multiforme (GBM).

The EGFR in Glioblastoma

Glioblastoma is the most common and lethal form of adult primary brain tumor and accounts for about 50% of all gliomas and 12-15% of all intracranial neoplasms (*165*). An estimated 17,400 patients are diagnosed with GBM in the United States every year (*166*). According to the world health organization classification system, glioblastoma is categorized as

the highest grade (Grade IV) astrocytoma, which is characterized by a high degree of cellularity, anaplasia, vascularization, proliferation, and necrosis (*167*). Clinical hallmarks of glioblastoma include its aggressive growth and inexorable recurrence despite multimodal therapy regiments that include surgery, radiation and chemotherapy (*168*). Despite aggressive treatment, the prognosis is dismal and median life expectancy of a patient with GBM ranges from 12-15 months (*169*).

Several different proteins have been identified that contribute to GBM formation, proliferation, and survival, and are currently being investigated as potential targets in treating GBM. Of these, receptor tyrosine kinases, including the EGFR, PDGFR, and hepatocyte growth factor receptor (c-MET), the PI3K pathway, as well as signaling pathways activated by the loss of PTEN (phosphatase and tensin homolog) and NF1 (neurofibromin 1), play central roles in the pathobiology of glioblastoma (*170*). Based on their unique molecular signatures, glioblastomas can be further divided into four subtypes (*171*). These four different subtypes include: the classical subtype, which is characterized by *EGFR* gene amplification; the mesenchymal subtype, which is characterized by NF1 loss; the pro-neural subtype, which has alterations in the PDGFR; and the neural subtype, which is typified by the expression of neuron markers such as *NEFL* and *GABRA1*.

One of the most common genetic alterations in primary GBM is the over-expression of the EGFR, which occurs in up to 60-90% of GBM (*165, 167, 170*). Whereas its expression in normal cells is estimated to be ~40,000-100,000 receptors per cell (*172*), in malignant tumors EGFR levels may reach 2 million per cell (*173*). Approximately 50% of GBMs reportedly have *EGFR* gene amplification, and these tumors invariably over-express the EGFR (*163, 174, 175*). However, EGFR over-expression without gene amplification events has been reported in up to

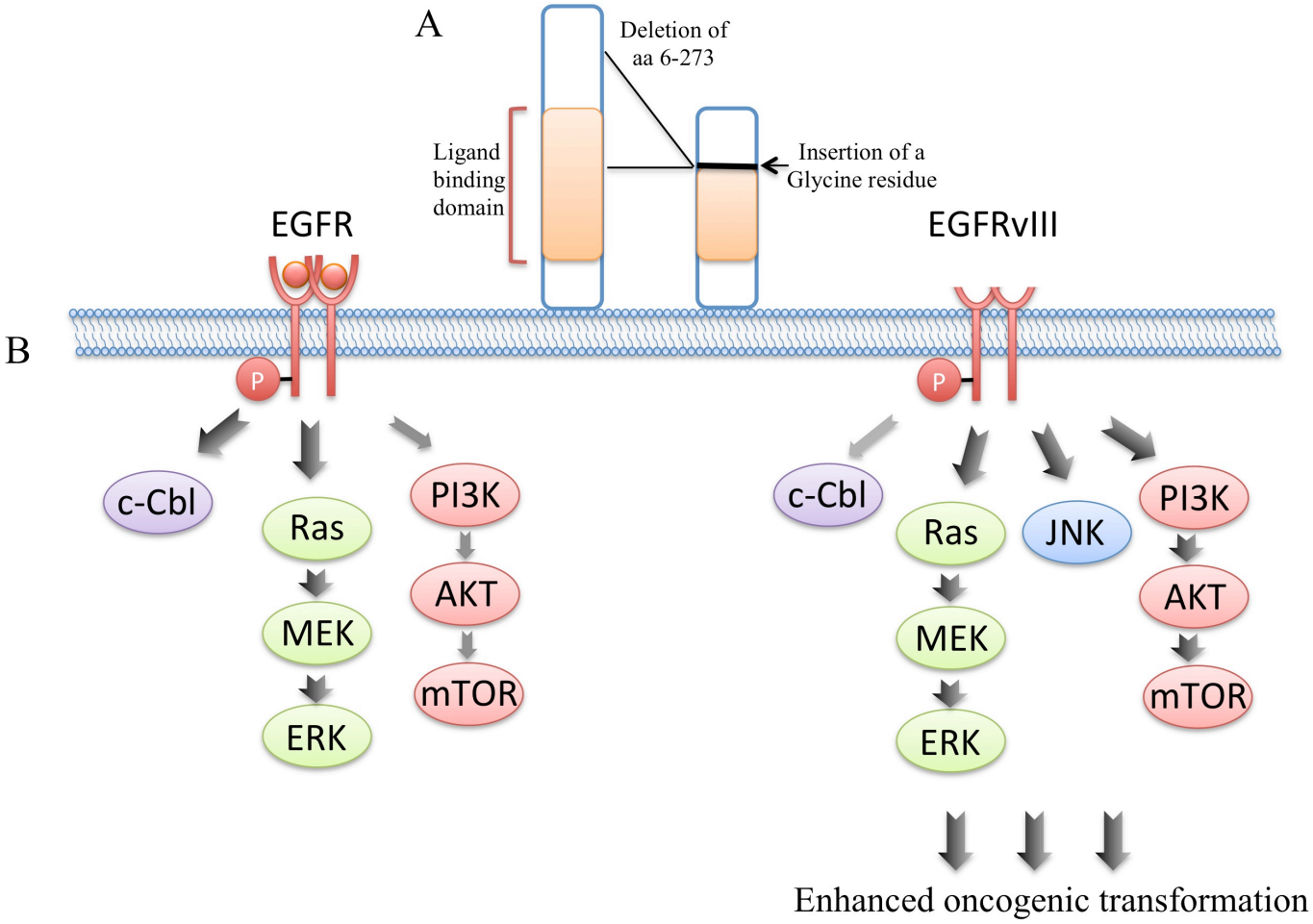
27% of GBM, suggesting that additional mechanisms that lead to EGFR over-expression must exist (*176*). Numerous studies have revealed a relationship between aberrant EGFR activity and the malignant nature of the glioblastoma cells (*177*). For example, EGFR over-expression promotes glioma cell proliferation and resistance to radiation therapy (*178*). Recent studies have also shown an unfavorable prognostic relationship between *EGFR* gene amplification and overall survival in GBM patients (*179*).

Besides gene amplification, activating mutations and truncations of the gene encoding the EGFR are also common mechanisms leading to excessive EGFR signaling activities in glioblastoma. One such example is the EGFR variant III (EGFRvIII). Approximately 50% of the glioblastomas that over-express wild type EGFR harbor the EGFRvIII mutant, which is generated by an in-frame deletion of exons 2-7, resulting in the loss of amino acids 6-273 in the extracellular domain of the EGFR (Figure 1.7) (*180, 181*). This truncated mutant receptor has a molecular mass of ~145 kDa compared with ~170 kDa for wild type EGFR, and is unable to bind to EGF due to deletion of most of the ligand-binding site in the extracellular domain (*182*). Despite this, however, the EGFRvIII exhibits constitutive tyrosine kinase activity and is defective in down-regulation (*183*). The expression of EGFRvIII has been linked to an extremely poor prognosis for GBM patients (*179, 184*). The ectopic expression of EGFRvIII in glioma cell lines induces constitutive activation of the Ras and PI3K signaling pathways (*185, 186*). Constitutively active c-Jun N-terminal kinase (JNK) is also found in EGFRvIII-positive cells but not in cells over-expressing wild type EGFR, indicating that the JNK pathway plays an important role in cell transformation by EGFRvIII (*187*). In addition, EGFRvIII has also been shown to enhance wild type EGFR signaling by up-regulating the expression of EGF in glioblastoma (*188*). Collectively, these signaling events lead to increased cell migration and

Figure 1.7. Comparison of the extracellular domain and the downstream signaling pathways between wild type EGFR and EGFRvIII.

(A) EGFRvIII is characterized by an in-frame deletion of exons 2–7 of the *EGFR* gene, which results in the loss of amino acids 6 to 273 and a novel glycine residue insertion in the extracellular domain. This mutation yields a form of EGFR that cannot associate with ligand but stays constitutively active.

(B) The major signaling pathways downstream of the ligand activated wild type EGFR and the constitutively active EGFRvIII include the PI3K and MAPK (Ras-MEK-ERK) signaling pathways. Binding of EGF to wild type EGFR initiates transient activation of the Ras-MEK-ERK pathway, and intermediate activation of the PI3K-Akt-mTOR pathway. Wild type EGFR phosphorylation also results in the recruitment of negative regulators such as c-Cbl, which promotes receptor internalization and signal termination. Activation of EGFRvIII also leads to activation of the Ras-MEK-ERK pathway. However, EGFRvIII activates the PI3K-Akt-mTOR pathway to a larger extent compared to wild type EGFR. EGFRvIII also constitutively activates JNK signaling pathway, which is not commonly observed with wild type EGFR. EGFRvIII also shows defective receptor internalization due to decreased complex formation with c-Cbl. Collectively, these effects lead to prolonged and excessive growth signals, which greatly promote oncogenic transformation. {Adapted from (*164*)}


A
Deletion of
aa 6-273
Ligand
binding
domain
Insertion of a
Glycine residue
B
EGFR
EGFRvIII
P
P
c-Cbl
Ras
MEK
ERK
PI3K
AKT
mTOR
c-Cbl
Ras
JNK
MEK
ERK
PI3K
AKT
mTOR
Enhanced oncogenic transformation

proliferation, and enhanced resistance to apoptosis induced by DNA-damaging agents, thus promoting glioblastoma progression (*189, 190*).

The identification of the EGFR as an oncogene has led to the development of therapies directed against this receptor. For example, monoclonal antibodies that bind to and prevent the activation of the EGFR, or small molecule inhibitors that block the tyrosine kinase activity of the EGFR, have been developed and proven useful in treating certain types of cancers (*133*). However, their use in the treatment of aggressive brain tumors has received limited success. Therefore, there continues to be an overriding need to identify other potential therapeutic targets for the treatment of these aggressive brain cancers.

Overview of the Thesis

Considering that tTG functions downstream of the EGFR and that tTG has been shown to promote several aspects cancer progression in other cancer cell types, we asked whether tTG could also contribute to the malignant transformation of glioblastoma cells. As described in Chapter 2 of this thesis, we found that, indeed, tTG is essential for the oncogenic phenotypes exhibited by these aggressive brain tumor cells. We showed that tTG expression is upregulated in high grade human brain tumor samples, and that knocking-down tTG expression in human glioblastoma cell lines blocked their oncogenic transformation. Moreover, we identified an unexpected pathway that tTG participates in EGFR signaling in these brain cancer cells. Specifically, tTG, in a conformation dependent manner, was able to block the action of the E3 Ubiquitin ligase c-Cbl and protect the EGFR from down-regulation, thus prolonging its signaling lifetime and promoting brain tumor progression.

From previous discoveries and data presented in Chapter 2, we can see that the two distinct conformational states of tTG differentially impact its functions. In Chapter 3, we set out to examine the molecular mechanisms that regulate the transition of tTG between its two functionally distinct states. We found that intramolecular interactions between the C-terminal tail of tTG and its catalytic core domain are important in maintaining tTG in its closed conformational state. Two pairs of hydrogen bonds, formed between residues N681 and K677 in the most C-terminal β-barrel domain and residues W254 and D434 localized in the catalytic domain, are required to stabilize tTG in its closed conformation. Disrupting these interactions caused tTG to adopt an open conformation that induces apoptosis in cells.

REFERENCES

1. Sarkar, N. K., Clarke, D. D., and Waelsch, H. (1957) An enzymically catalyzed incorporation of amines into proteins, *Biochimica et biophysica acta 25*, 451-452.

2. Verma, A., Wang, H., Manavathi, B., Fok, J. Y., Mann, A. P., Kumar, R., and Mehta, K. (2006) Increased expression of tissue transglutaminase in pancreatic ductal adenocarcinoma and its implications in drug resistance and metastasis, *Cancer research 66*, 10525-10533.

3. Hwang, J. Y., Mangala, L. S., Fok, J. Y., Lin, Y. G., Merritt, W. M., Spannuth, W. A., Nick, A. M., Fiterman, D. J., Vivas-Mejia, P. E., Deavers, M. T., Coleman, R. L., Lopez-Berestein, G., Mehta, K., and Sood, A. K. (2008) Clinical and biological significance of tissue transglutaminase in ovarian carcinoma, *Cancer research 68*, 5849-5858.

4. Singer, C. F., Hudelist, G., Walter, I., Rueckliniger, E., Czerwenka, K., Kubista, E., and Huber, A. V. (2006) Tissue array-based expression of transglutaminase-2 in human breast and ovarian cancer, *Clinical & experimental metastasis 23*, 33-39.

5. Satpathy, M., Cao, L., Pincheira, R., Emerson, R., Bigsby, R., Nakshatri, H., and Matei, D. (2007) Enhanced peritoneal ovarian tumor dissemination by tissue transglutaminase, *Cancer research 67*, 7194-7202.

6. Li, B., Cerione, R. A., and Antonyak, M. (2011) Tissue transglutaminase and its role in human cancer progression, *Advances in enzymology and related areas of molecular biology 78*, 247-293.

7. Lorand, L., and Graham, R. M. (2003) Transglutaminases: crosslinking enzymes with pleiotropic functions, *Nature reviews. Molecular cell biology 4*, 140-156.

8. Folk, J. E. (1980) Transglutaminases, *Annu Rev Biochem 49*, 517-531.

9. Greenberg, C. S., Birckbichler, P. J., and Rice, R. H. (1991) Transglutaminases: multifunctional cross-linking enzymes that stabilize tissues, *Faseb J 5*, 3071-3077.

10. Facchiano, A., and Facchiano, F. (2009) Transglutaminases and their substrates in biology and human diseases: 50 years of growing, *Amino acids 36*, 599-614.

11. Sane, D. C., Kontos, J. L., and Greenberg, C. S. (2007) Roles of transglutaminases in cardiac and vascular diseases, *Frontiers in bioscience : a journal and virtual library 12*, 2530-2545.

12. Hoffner, G., Vanhoutteghem, A., Andre, W., and Djian, P. (2011) Transglutaminase in epidermis and neurological disease or what makes a good cross-linking substrate, *Advances in enzymology and related areas of molecular biology 78*, 97-160.

13. Gentile, V. (2011) Physiopathological roles of human transglutaminase 2, *Advances in enzymology and related areas of molecular biology 78*, 47-95.

14. Muszbek, L., Yee, V. C., and Hevessy, Z. (1999) Blood coagulation factor XIII: structure and function, *Thrombosis research 94*, 271-305.

15. Ichinose, A. (2001) Physiopathology and regulation of factor XIII, *Thrombosis and haemostasis 86*, 57-65.

16. Candi, E., Schmidt, R., and Melino, G. (2005) The cornified envelope: a model of cell death in the skin, *Nature reviews. Molecular cell biology 6*, 328-340.

17. Hennies, H. C., Kuster, W., Wiebe, V., Krebsova, A., and Reis, A. (1998) Genotype/phenotype correlation in autosomal recessive lamellar ichthyosis, *American journal of human genetics 62*, 1052-1061.

18. Huber, M., Rettler, I., Bernasconi, K., Frenk, E., Lavrijsen, S. P., Ponec, M., Bon, A., Lautenschlager, S., Schorderet, D. F., and Hohl, D. (1995) Mutations of keratinocyte transglutaminase in lamellar ichthyosis, *Science 267*, 525-528.

19. Nurminskaya, M. V., and Belkin, A. M. (2012) Cellular functions of tissue transglutaminase, *International review of cell and molecular biology 294*, 1-97.

20. Casadio, R., Polverini, E., Mariani, P., Spinozzi, F., Carsughi, F., Fontana, A., Polverino de Laureto, P., Matteucci, G., and Bergamini, C. M. (1999) The structural basis for the regulation of tissue transglutaminase by calcium ions, *European journal of biochemistry / FEBS 262*, 672-679.

21. Liu, S., Cerione, R. A., and Clardy, J. (2002) Structural basis for the guanine nucleotide-binding activity of tissue transglutaminase and its regulation of transamidation activity, *Proceedings of the National Academy of Sciences of the United States of America 99*, 2743-2747.

22. Murthy, S. N., Iismaa, S., Begg, G., Freymann, D. M., Graham, R. M., and Lorand, L. (2002) Conserved tryptophan in the core domain of transglutaminase is essential for catalytic activity, *Proceedings of the National Academy of Sciences of the United States of America 99*, 2738-2742.

23. Li, B., Antonyak, M. A., Druso, J. E., Cheng, L., Nikitin, A. Y., and Cerione, R. A. (2010) EGF potentiated oncogenesis requires a tissue transglutaminase-dependent signaling pathway leading to Src activation, *Proceedings of the National Academy of Sciences of the United States of America 107*, 1408-1413.

24. Aeschlimann, D., Kaupp, O., and Paulsson, M. (1995) Transglutaminase-catalyzed matrix cross-linking in differentiating cartilage: identification of osteonectin as a major glutaminyl substrate, *The Journal of cell biology 129*, 881-892.

25. Haroon, Z. A., Hettasch, J. M., Lai, T. S., Dewhirst, M. W., and Greenberg, C. S. (1999) Tissue transglutaminase is expressed, active, and directly involved in rat dermal wound healing and angiogenesis, *Faseb J 13*, 1787-1795.

26. Antonyak, M. A., Singh, U. S., Lee, D. A., Boehm, J. E., Combs, C., Zgola, M. M., Page, R. L., and Cerione, R. A. (2001) Effects of tissue transglutaminase on retinoic acid-induced cellular differentiation and protection against apoptosis, *The Journal of biological chemistry 276*, 33582-33587.

27. Boehm, J. E., Singh, U., Combs, C., Antonyak, M. A., and Cerione, R. A. (2002) Tissue transglutaminase protects against apoptosis by modifying the tumor suppressor protein p110 Rb, *The Journal of biological chemistry 277*, 20127-20130.

28. Fujita, K., Shibayama, K., Yamauchi, M., Kato, T., Ando, M., Takahashi, H., Iritani, K., Yoshimoto, N., and Nagata, Y. (1998) Alteration of enzymatic activities implicating neuronal degeneration in the spinal cord of the motor neuron degeneration mouse during postnatal development, *Neurochem Res 23*, 557-562.

29. Verderio, E. A., Johnson, T., and Griffin, M. (2004) Tissue transglutaminase in normal and abnormal wound healing: review article, *Amino acids 26*, 387-404.

30. Tee, A. E., Marshall, G. M., Liu, P. Y., Xu, N., Haber, M., Norris, M. D., Iismaa, S. E., and Liu, T. (2009) The opposing effects of two tissue transglutaminase protein isoforms in neuroblastoma cell differentiation, *The Journal of biological chemistry*.

31. Gilman, A. G. (1987) G proteins: transducers of receptor-generated signals, *Annu Rev Biochem 56*, 615-649.

32. Nakaoka, H., Perez, D. M., Baek, K. J., Das, T., Husain, A., Misono, K., Im, M. J., and Graham, R. M. (1994) Gh: a GTP-binding protein with transglutaminase activity and receptor signaling function, *Science 264*, 1593-1596.

33. Lee, M. Y., Chung, S., Bang, H. W., Baek, K. J., and Uhm, D. (1997) Modulation of large conductance Ca2+-activated K+ channel by Galphah (transglutaminase II) in the vascular smooth muscle cell, *Pflugers Arch 433*, 671-673.

34. Sprang, S. R. (1997) G protein mechanisms: insights from structural analysis, *Annu Rev Biochem 66*, 639-678.

35. Phillips, M. J., Calero, G., Chan, B., Ramachandran, S., and Cerione, R. A. (2008) Effector proteins exert an important influence on the signaling-active state of the small GTPase Cdc42, *The Journal of biological chemistry 283*, 14153-14164.

36. Datta, S., Antonyak, M. A., and Cerione, R. A. (2007) GTP-binding-defective forms of tissue transglutaminase trigger cell death, *Biochemistry 46*, 14819-14829.

37. Achyuthan, K. E., and Greenberg, C. S. (1987) Identification of a guanosine triphosphate-binding site on guinea pig liver transglutaminase. Role of GTP and calcium ions in modulating activity, *The Journal of biological chemistry 262*, 1901-1906.

38. Datta, S., Antonyak, M. A., and Cerione, R. A. (2006) Importance of Ca(2+)-dependent transamidation activity in the protection afforded by tissue transglutaminase against doxorubicin-induced apoptosis, *Biochemistry 45*, 13163-13174.

39. Begg, G. E., Holman, S. R., Stokes, P. H., Matthews, J. M., Graham, R. M., and Iismaa, S. E. (2006) Mutation of a critical arginine in the GTP-binding site of transglutaminase 2 disinhibits intracellular cross-linking activity, *The Journal of biological chemistry 281*, 12603-12609.

40. Begg, G. E., Carrington, L., Stokes, P. H., Matthews, J. M., Wouters, M. A., Husain, A., Lorand, L., Iismaa, S. E., and Graham, R. M. (2006) Mechanism of allosteric regulation of transglutaminase 2 by GTP, *Proceedings of the National Academy of Sciences of the United States of America 103*, 19683-19688.

41. Pinkas, D. M., Strop, P., Brunger, A. T., and Khosla, C. (2007) Transglutaminase 2 undergoes a large conformational change upon activation, *PLoS Biol 5*, e327.

42. Ahvazi, B., Kim, H. C., Kee, S. H., Nemes, Z., and Steinert, P. M. (2002) Three-dimensional structure of the human transglutaminase 3 enzyme: binding of calcium ions changes structure for activation, *The EMBO journal 21*, 2055-2067.

43. Antonyak, M. A., Miller, A. M., Jansen, J. M., Boehm, J. E., Balkman, C. E., Wakshlag, J. J., Page, R. L., and Cerione, R. A. (2004) Augmentation of tissue transglutaminase expression and activation by epidermal growth factor inhibit doxorubicin-induced apoptosis in human breast cancer cells, *The Journal of biological chemistry 279*, 41461-41467.

44. Giandomenico, V., Lancillotti, F., Fiorucci, G., Percario, Z. A., Rivabene, R., Malorni, W., Affabris, E., and Romeo, G. (1997) Retinoic acid and IFN inhibition of cell proliferation is associated with apoptosis in squamous carcinoma cell lines: role of IRF-1 and TGase II-dependent pathways, *Cell growth & differentiation : the molecular biology journal of the American Association for Cancer Research 8*, 91-100.

45. Johnson, K., Hashimoto, S., Lotz, M., Pritzker, K., and Terkeltaub, R. (2001) Interleukin-1 induces pro-mineralizing activity of cartilage tissue transglutaminase and factor XIIIa, *The American journal of pathology 159*, 149-163.

46. Jang, G. Y., Jeon, J. H., Cho, S. Y., Shin, D. M., Kim, C. W., Jeong, E. M., Bae, H. C., Kim, T. W., Lee, S. H., Choi, Y., Lee, D. S., Park, S. C., and Kim, I. G. (2010) Transglutaminase 2 suppresses apoptosis by modulating caspase 3 and NF-kappaB activity in hypoxic tumor cells, *Oncogene 29*, 356-367.

47. Chiocca, E. A., Davies, P. J., and Stein, J. P. (1989) Regulation of tissue transglutaminase gene expression as a molecular model for retinoid effects on proliferation and differentiation, *Journal of cellular biochemistry 39*, 293-304.

48. Piacentini, M., Annicchiarico-Petruzzelli, M., Oliverio, S., Piredda, L., Biedler, J. L., and Melino, E. (1992) Phenotype-specific "tissue" transglutaminase regulation in human neuroblastoma cells in response to retinoic acid: correlation with cell death by apoptosis, *International journal of cancer. Journal international du cancer 52*, 271-278.

49. Chambon, P. (1996) A decade of molecular biology of retinoic acid receptors, *Faseb J 10*, 940-954.

50. Nagy, L., Saydak, M., Shipley, N., Lu, S., Basilion, J. P., Yan, Z. H., Syka, P., Chandraratna, R. A., Stein, J. P., Heyman, R. A., and Davies, P. J. (1996) Identification and characterization of a versatile retinoid response element (retinoic acid receptor response element-retinoid X receptor response element) in the mouse tissue transglutaminase gene promoter, *The Journal of biological chemistry 271*, 4355-4365.

51. Mirza, A., Liu, S. L., Frizell, E., Zhu, J., Maddukuri, S., Martinez, J., Davies, P., Schwarting, R., Norton, P., and Zern, M. A. (1997) A role for tissue transglutaminase in hepatic injury and fibrogenesis, and its regulation by NF-kappaB, *The American journal of physiology 272*, G281-288.

52. Kuncio, G. S., Tsyganskaya, M., Zhu, J., Liu, S. L., Nagy, L., Thomazy, V., Davies, P. J., and Zern, M. A. (1998) TNF-alpha modulates expression of the tissue transglutaminase gene in liver cells, *The American journal of physiology 274*, G240-245.

53. Li, X., and Stark, G. R. (2002) NFkappaB-dependent signaling pathways, *Experimental hematology 30*, 285-296.

54. Mehta, K., Kumar, A., and Kim, H. I. (2010) Transglutaminase 2: a multi-tasking protein in the complex circuitry of inflammation and cancer, *Biochemical pharmacology 80*, 1921-1929.

55. Zhang, R., Tremblay, T. L., McDermid, A., Thibault, P., and Stanimirovic, D. (2003) Identification of differentially expressed proteins in human glioblastoma cell lines and tumors, *Glia 42*, 194-208.

56. Antonyak, M. A., Li, B., Regan, A. D., Feng, Q., Dusaban, S. S., and Cerione, R. A. (2009) Tissue transglutaminase is an essential participant in the epidermal growth factor-stimulated signaling pathway leading to cancer cell migration and invasion, *The Journal of biological chemistry 284*, 17914-17925.

57. Lesort, M., Attanavanich, K., Zhang, J., and Johnson, G. V. (1998) Distinct nuclear localization and activity of tissue transglutaminase, *The Journal of biological chemistry 273*, 11991-11994.

58. Chowdhury, Z. A., Barsigian, C., Chalupowicz, G. D., Bach, T. L., Garcia-Manero, G., and Martinez, J. (1997) Colocalization of tissue transglutaminase and stress fibers in human vascular smooth muscle cells and human umbilical vein endothelial cells, *Experimental cell research 231*, 38-49.

59. Kiraly, R., Demeny, M., and Fesus, L. (2011) Protein transamidation by transglutaminase 2 in cells: a disputed Ca2+-dependent action of a multifunctional protein, *The FEBS journal 278*, 4717-4739.

60. Balajthy, Z., Csomos, K., Vamosi, G., Szanto, A., Lanotte, M., and Fesus, L. (2006) Tissue-transglutaminase contributes to neutrophil granulocyte differentiation and functions, *Blood 108*, 2045-2054.

61. Dardik, R., and Inbal, A. (2006) Complex formation between tissue transglutaminase II (tTG) and vascular endothelial growth factor receptor 2 (VEGFR-2): proposed mechanism for modulation of endothelial cell response to VEGF, *Experimental cell research 312*, 2973-2982.

62. Peng, X., Zhang, Y., Zhang, H., Graner, S., Williams, J. F., Levitt, M. L., and Lokshin, A. (1999) Interaction of tissue transglutaminase with nuclear transport protein importin-alpha3, *FEBS letters 446*, 35-39.

63. McConoughey, S. J., Basso, M., Niatsetskaya, Z. V., Sleiman, S. F., Smirnova, N. A., Langley, B. C., Mahishi, L., Cooper, A. J., Antonyak, M. A., Cerione, R. A., Li, B., Starkov, A., Chaturvedi, R. K., Beal, M. F., Coppola, G., Geschwind, D. H., Ryu, H., Xia, L., Iismaa, S. E., Pallos, J., Pasternack, R., Hils, M., Fan, J., Raymond, L. A., Marsh, J. L., Thompson, L. M., and Ratan, R. R. (2010) Inhibition of transglutaminase 2 mitigates transcriptional dysregulation in models of Huntington disease, *EMBO molecular medicine 2*, 349-370.

64. Ballestar, E., Abad, C., and Franco, L. (1996) Core histones are glutaminyl substrates for tissue transglutaminase, *The Journal of biological chemistry 271*, 18817-18824.

65. Ballestar, E., Boix-Chornet, M., and Franco, L. (2001) Conformational changes in the nucleosome followed by the selective accessibility of histone glutamines in the transglutaminase reaction: effects of ionic strength, *Biochemistry 40*, 1922-1929.

66. Herwig, S., and Strauss, M. (1997) The retinoblastoma protein: a master regulator of cell cycle, differentiation and apoptosis, *European journal of biochemistry / FEBS 246*, 581-601.

67. Gottlieb, E., and Oren, M. (1998) p53 facilitates pRb cleavage in IL-3-deprived cells: novel pro-apoptotic activity of p53, *The EMBO journal 17*, 3587-3596.

68. Lee, E. Y., Chang, C. Y., Hu, N., Wang, Y. C., Lai, C. C., Herrup, K., Lee, W. H., and Bradley, A. (1992) Mice deficient for Rb are nonviable and show defects in neurogenesis and haematopoiesis, *Nature 359*, 288-294.

69. Verderio, E., Nicholas, B., Gross, S., and Griffin, M. (1998) Regulated expression of tissue transglutaminase in Swiss 3T3 fibroblasts: effects on the processing of fibronectin, cell attachment, and cell death, *Experimental cell research 239*, 119-138.

70. Aeschlimann, D., and Thomazy, V. (2000) Protein crosslinking in assembly and remodelling of extracellular matrices: the role of transglutaminases, *Connective tissue research 41*, 1-27.

71. Nelea, V., Nakano, Y., and Kaartinen, M. T. (2008) Size distribution and molecular associations of plasma fibronectin and fibronectin crosslinked by transglutaminase 2, *The protein journal 27*, 223-233.

72. Spurlin, T. A., Bhadriraju, K., Chung, K. H., Tona, A., and Plant, A. L. (2009) The treatment of collagen fibrils by tissue transglutaminase to promote vascular smooth muscle cell contractile signaling, *Biomaterials 30*, 5486-5496.

73. Belkin, A. M., Tsurupa, G., Zemskov, E., Veklich, Y., Weisel, J. W., and Medved, L. (2005) Transglutaminase-mediated oligomerization of the fibrin(ogen) alphaC domains promotes integrin-dependent cell adhesion and signaling, *Blood 105*, 3561-3568.

74. Worthington, J. J., Klementowicz, J. E., and Travis, M. A. (2011) TGFbeta: a sleeping giant awoken by integrins, *Trends in biochemical sciences 36*, 47-54.

75. Roberts, A. B., McCune, B. K., and Sporn, M. B. (1992) TGF-beta: regulation of extracellular matrix, *Kidney international 41*, 557-559.

76. Kojima, S., Nara, K., and Rifkin, D. B. (1993) Requirement for transglutaminase in the activation of latent transforming growth factor-beta in bovine endothelial cells, *The Journal of cell biology 121*, 439-448.

77. Verderio, E., Gaudry, C., Gross, S., Smith, C., Downes, S., and Griffin, M. (1999) Regulation of cell surface tissue transglutaminase: effects on matrix storage of latent transforming growth factor-beta binding protein-1, *The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society 47*, 1417-1432.

78. Akimov, S. S., Krylov, D., Fleischman, L. F., and Belkin, A. M. (2000) Tissue transglutaminase is an integrin-binding adhesion coreceptor for fibronectin, *The Journal of cell biology 148*, 825-838.

79. Belkin, A. M. (2011) Extracellular TG2: emerging functions and regulation, *The FEBS journal 278*, 4704-4716.

80. Zemskov, E. A., Janiak, A., Hang, J., Waghray, A., and Belkin, A. M. (2006) The role of tissue transglutaminase in cell-matrix interactions, *Frontiers in bioscience : a journal and virtual library 11*, 1057-1076.

81. Zemskov, E. A., Mikhailenko, I., Smith, E. P., and Belkin, A. M. (2012) Tissue transglutaminase promotes PDGF/PDGFR-mediated signaling and responses in vascular smooth muscle cells, *Journal of cellular physiology 227*, 2089-2096.

82. Zemskov, E. A., Loukinova, E., Mikhailenko, I., Coleman, R. A., Strickland, D. K., and Belkin, A. M. (2009) Regulation of platelet-derived growth factor receptor function by integrin-associated cell surface transglutaminase, *The Journal of biological chemistry 284*, 16693-16703.

83. Wang, Z., and Griffin, M. (2012) TG2, a novel extracellular protein with multiple functions, *Amino acids 42*, 939-949.

84. Gentile, V., Saydak, M., Chiocca, E. A., Akande, O., Birckbichler, P. J., Lee, K. N., Stein, J. P., and Davies, P. J. (1991) Isolation and characterization of cDNA clones to mouse macrophage and human endothelial cell tissue transglutaminases, *The Journal of biological chemistry 266*, 478-483.

85. Antonyak, M. A., Li, B., Boroughs, L. K., Johnson, J. L., Druso, J. E., Bryant, K. L., Holowka, D. A., and Cerione, R. A. (2011) Cancer cell-derived microvesicles induce transformation by transferring tissue transglutaminase and fibronectin to recipient cells, *Proceedings of the National Academy of Sciences of the United States of America 108*, 4852-4857.

86. Milano, S. K., Kwon, W., Pereira, R., Antonyak, M. A., and Cerione, R. A. (2012) Characterization of a Novel Activated Ran GTPase Mutant and Its Ability to Induce Cellular Transformation, *The Journal of biological chemistry 287*, 24955-24966.

87. Skog, J., Wurdinger, T., van Rijn, S., Meijer, D. H., Gainche, L., Sena-Esteves, M., Curry, W. T., Jr., Carter, B. S., Krichevsky, A. M., and Breakefield, X. O. (2008) Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers, *Nature cell biology 10*, 1470-1476.

88. Cocucci, E., Racchetti, G., and Meldolesi, J. (2009) Shedding microvesicles: artefacts no more, *Trends in cell biology 19*, 43-51.

89. Muralidharan-Chari, V., Clancy, J. W., Sedgwick, A., and D'Souza-Schorey, C. (2010) Microvesicles: mediators of extracellular communication during cancer progression, *Journal of cell science 123*, 1603-1611.

90. Al-Nedawi, K., Meehan, B., Micallef, J., Lhotak, V., May, L., Guha, A., and Rak, J. (2008) Intercellular transfer of the oncogenic receptor EGFRvIII by microvesicles derived from tumour cells, *Nature cell biology 10*, 619-624.

91. Piacentini, M., Farrace, M. G., Piredda, L., Matarrese, P., Ciccosanti, F., Falasca, L., Rodolfo, C., Giammarioli, A. M., Verderio, E., Griffin, M., and Malorni, W. (2002) Transglutaminase over-expression sensitizes neuronal cell lines to apoptosis by increasing mitochondrial membrane potential and cellular oxidative stress, *Journal of neurochemistry 81*, 1061-1072.

92. Rodolfo, C., Mormone, E., Matarrese, P., Ciccosanti, F., Farrace, M. G., Garofano, E., Piredda, L., Fimia, G. M., Malorni, W., and Piacentini, M. (2004) Tissue transglutaminase is a multifunctional BH3-only protein, *The Journal of biological chemistry 279*, 54783-54792.

93. Nanda, N., Iismaa, S. E., Owens, W. A., Husain, A., Mackay, F., and Graham, R. M. (2001) Targeted inactivation of Gh/tissue transglutaminase II, *The Journal of biological chemistry 276*, 20673-20678.

94. De Laurenzi, V., and Melino, G. (2001) Gene disruption of tissue transglutaminase, *Molecular and cellular biology 21*, 148-155.

95. Szondy, Z., Mastroberardino, P. G., Varadi, J., Farrace, M. G., Nagy, N., Bak, I., Viti, I., Wieckowski, M. R., Melino, G., Rizzuto, R., Tosaki, A., Fesus, L., and Piacentini, M. (2006) Tissue transglutaminase (TG2) protects cardiomyocytes against ischemia/reperfusion injury by regulating ATP synthesis, *Cell death and differentiation 13*, 1827-1829.

96. Lesort, M., Tucholski, J., Miller, M. L., and Johnson, G. V. (2000) Tissue transglutaminase: a possible role in neurodegenerative diseases, *Progress in neurobiology 61*, 439-463.

97. Ientile, R., Caccamo, D., Marciano, M. C., Curro, M., Mannucci, C., Campisi, A., and Calapai, G. (2004) Transglutaminase activity and transglutaminase mRNA transcripts in gerbil brain ischemia, *Neuroscience letters 363*, 173-177.

98. Grosso, H., and Mouradian, M. M. (2012) Transglutaminase 2: biology, relevance to neurodegenerative diseases and therapeutic implications, *Pharmacology & therapeutics 133*, 392-410.

99. Jeitner, T. M., Pinto, J. T., Krasnikov, B. F., Horswill, M., and Cooper, A. J. (2009) Transglutaminases and neurodegeneration, *Journal of neurochemistry 109 Suppl 1*, 160-166.

100. Olzmann, J. A., Li, L., and Chin, L. S. (2008) Aggresome formation and neurodegenerative diseases: therapeutic implications, *Current medicinal chemistry 15*, 47-60.

101. Ross, C. A., and Poirier, M. A. (2004) Protein aggregation and neurodegenerative disease, *Nature medicine 10 Suppl*, S10-17.

102. Jeitner, T. M., Muma, N. A., Battaile, K. P., and Cooper, A. J. (2009) Transglutaminase activation in neurodegenerative diseases, *Future neurology 4*, 449-467.

103. Cooper, A. J., Jeitner, T. M., Gentile, V., and Blass, J. P. (2002) Cross linking of polyglutamine domains catalyzed by tissue transglutaminase is greatly favored with pathological-length repeats: does transglutaminase activity play a role in (CAG)(n)/Q(n)-expansion diseases?, *Neurochemistry international 40*, 53-67.

104. Martindale, D., Hackam, A., Wieczorek, A., Ellerby, L., Wellington, C., McCutcheon, K., Singaraja, R., Kazemi-Esfarjani, P., Devon, R., Kim, S. U., Bredesen, D. E., Tufaro, F., and Hayden, M. R. (1998) Length of huntingtin and its polyglutamine tract influences localization and frequency of intracellular aggregates, *Nature genetics 18*, 150-154.

105. Sawa, A., Wiegand, G. W., Cooper, J., Margolis, R. L., Sharp, A. H., Lawler, J. F., Jr., Greenamyre, J. T., Snyder, S. H., and Ross, C. A. (1999) Increased apoptosis of Huntington disease lymphoblasts associated with repeat length-dependent mitochondrial depolarization, *Nature medicine 5*, 1194-1198.

106. Kennedy, L., Evans, E., Chen, C. M., Craven, L., Detloff, P. J., Ennis, M., and Shelbourne, P. F. (2003) Dramatic tissue-specific mutation length increases are an early molecular event in Huntington disease pathogenesis, *Human molecular genetics 12*, 3359-3367.

107. Cooper, A. J., Sheu, K. F., Burke, J. R., Onodera, O., Strittmatter, W. J., Roses, A. D., and Blass, J. P. (1997) Polyglutamine domains are substrates of tissue transglutaminase: does transglutaminase play a role in expanded CAG/poly-Q neurodegenerative diseases?, *Journal of neurochemistry 69*, 431-434.

108. de Cristofaro, T., Affaitati, A., Cariello, L., Avvedimento, E. V., and Varrone, S. (1999) The length of polyglutamine tract, its level of expression, the rate of degradation, and the transglutaminase activity influence the formation of intracellular aggregates, *Biochemical and biophysical research communications 260*, 150-158.

109. Karpuj, M. V., Garren, H., Slunt, H., Price, D. L., Gusella, J., Becher, M. W., and Steinman, L. (1999) Transglutaminase aggregates huntingtin into nonamyloidogenic polymers, and its enzymatic activity increases in Huntington's disease brain nuclei, *Proceedings of the National Academy of Sciences of the United States of America 96*, 7388-7393.

110. Zainelli, G. M., Ross, C. A., Troncoso, J. C., and Muma, N. A. (2003) Transglutaminase cross-links in intranuclear inclusions in Huntington disease, *Journal of neuropathology and experimental neurology 62*, 14-24.

111. Karpuj, M. V., Becher, M. W., Springer, J. E., Chabas, D., Youssef, S., Pedotti, R., Mitchell, D., and Steinman, L. (2002) Prolonged survival and decreased abnormal movements in transgenic model of Huntington disease, with administration of the transglutaminase inhibitor cystamine, *Nature medicine 8*, 143-149.

112. Dedeoglu, A., Kubilus, J. K., Jeitner, T. M., Matson, S. A., Bogdanov, M., Kowall, N. W., Matson, W. R., Cooper, A. J., Ratan, R. R., Beal, M. F., Hersch, S. M., and Ferrante, R. J. (2002) Therapeutic effects of cystamine in a murine model of Huntington's disease, *The Journal of neuroscience : the official journal of the Society for Neuroscience 22*, 8942-8950.

113. Mastroberardino, P. G., Iannicola, C., Nardacci, R., Bernassola, F., De Laurenzi, V., Melino, G., Moreno, S., Pavone, F., Oliverio, S., Fesus, L., and Piacentini, M. (2002)

'Tissue' transglutaminase ablation reduces neuronal death and prolongs survival in a mouse model of Huntington's disease, *Cell death and differentiation 9*, 873-880.

114. Appelt, D. M., Kopen, G. C., Boyne, L. J., and Balin, B. J. (1996) Localization of transglutaminase in hippocampal neurons: implications for Alzheimer's disease, *The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society 44*, 1421-1427.

115. Antonyak, M. A., Jansen, J. M., Miller, A. M., Ly, T. K., Endo, M., and Cerione, R. A. (2006) Two isoforms of tissue transglutaminase mediate opposing cellular fates, *Proceedings of the National Academy of Sciences of the United States of America 103*, 18609-18614.

116. Citron, B. A., SantaCruz, K. S., Davies, P. J., and Festoff, B. W. (2001) Intron-exon swapping of transglutaminase mRNA and neuronal Tau aggregation in Alzheimer's disease, *The Journal of biological chemistry 276*, 3295-3301.

117. Citron, B. A., Suo, Z., SantaCruz, K., Davies, P. J., Qin, F., and Festoff, B. W. (2002) Protein crosslinking, tissue transglutaminase, alternative splicing and neurodegeneration, *Neurochemistry international 40*, 69-78.

118. Boroughs, L. K., Antonyak, M. A., Johnson, J. L., and Cerione, R. A. (2011) A unique role for heat shock protein 70 and its binding partner tissue transglutaminase in cancer cell migration, *The Journal of biological chemistry 286*, 37094-37107.

119. Le Clainche, C., and Carlier, M. F. (2008) Regulation of actin assembly associated with protrusion and adhesion in cell migration, *Physiological reviews 88*, 489-513.

120. Jiang, D., Ying, W., Lu, Y., Wan, J., Zhai, Y., Liu, W., Zhu, Y., Qiu, Z., Qian, X., and He, F. (2003) Identification of metastasis-associated proteins by proteomic analysis and functional exploration of interleukin-18 in metastasis, *Proteomics 3*, 724-737.

121. Jiang, W. G., Ablin, R., Douglas-Jones, A., and Mansel, R. E. (2003) Expression of transglutaminases in human breast cancer and their possible clinical significance, *Oncology reports 10*, 2039-2044.

122. Kumar, A., Xu, J., Brady, S., Gao, H., Yu, D., Reuben, J., and Mehta, K. (2010) Tissue transglutaminase promotes drug resistance and invasion by inducing mesenchymal transition in mammary epithelial cells, *PloS one 5*, e13390.

123. Yang, J., and Weinberg, R. A. (2008) Epithelial-mesenchymal transition: at the crossroads of development and tumor metastasis, *Developmental cell 14*, 818-829.

124. Lowe, S. W., and Lin, A. W. (2000) Apoptosis in cancer, *Carcinogenesis 21*, 485-495.

125. Fesus, L., and Szondy, Z. (2005) Transglutaminase 2 in the balance of cell death and survival, *FEBS letters 579*, 3297-3302.

126. Cho, S. Y., Lee, J. H., Bae, H. D., Jeong, E. M., Jang, G. Y., Kim, C. W., Shin, D. M., Jeon, J. H., and Kim, I. G. (2010) Transglutaminase 2 inhibits apoptosis induced by calcium- overload through down-regulation of Bax, *Experimental & molecular medicine 42*, 639-650.

127. Verma, A., and Mehta, K. (2007) Tissue transglutaminase-mediated chemoresistance in cancer cells, *Drug resistance updates : reviews and commentaries in antimicrobial and anticancer chemotherapy 10*, 144-151.

128. Mehta, K., Fok, J., Miller, F. R., Koul, D., and Sahin, A. A. (2004) Prognostic significance of tissue transglutaminase in drug resistant and metastatic breast cancer, *Clinical cancer research : an official journal of the American Association for Cancer Research 10*, 8068-8076.

129. Herman, J. F., Mangala, L. S., and Mehta, K. (2006) Implications of increased tissue transglutaminase (TG2) expression in drug-resistant breast cancer (MCF-7) cells, *Oncogene 25*, 3049-3058.

130. Mann, A. P., Verma, A., Sethi, G., Manavathi, B., Wang, H., Fok, J. Y., Kunnumakkara, A. B., Kumar, R., Aggarwal, B. B., and Mehta, K. (2006) Over-expression of tissue transglutaminase leads to constitutive activation of nuclear factor-kappaB in cancer cells: delineation of a novel pathway, *Cancer research 66*, 8788-8795.

131. Verma, A., and Mehta, K. (2007) Transglutaminase-mediated activation of nuclear transcription factor-kappaB in cancer cells: a new therapeutic opportunity, *Current cancer drug targets 7*, 559-565.

132. Jove, R., and Hanafusa, H. (1987) Cell transformation by the viral src oncogene, *Annual review of cell biology 3*, 31-56.

133. Seshacharyulu, P., Ponnusamy, M. P., Haridas, D., Jain, M., Ganti, A. K., and Batra, S. K. (2012) Targeting the EGFR signaling pathway in cancer therapy, *Expert opinion on therapeutic targets 16*, 15-31.

134. Rasheed, B. K., Wiltshire, R. N., Bigner, S. H., and Bigner, D. D. (1999) Molecular pathogenesis of malignant gliomas, *Current opinion in oncology 11*, 162-167.

135. Ullrich, A., Coussens, L., Hayflick, J. S., Dull, T. J., Gray, A., Tam, A. W., Lee, J., Yarden, Y., Libermann, T. A., Schlessinger, J., and et al. (1984) Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermoid carcinoma cells, *Nature 309*, 418-425.

136. Olayioye, M. A., Neve, R. M., Lane, H. A., and Hynes, N. E. (2000) The ErbB signaling network: receptor heterodimerization in development and cancer, *The EMBO journal 19*, 3159-3167.

137. Yarden, Y., and Sliwkowski, M. X. (2001) Untangling the ErbB signalling network, *Nature reviews. Molecular cell biology 2*, 127-137.

138. Citri, A., and Yarden, Y. (2006) EGF-ERBB signalling: towards the systems level, *Nature reviews. Molecular cell biology 7*, 505-516.

139. Wells, A. (1999) EGF receptor, *The international journal of biochemistry & cell biology 31*, 637-643.

140. Ferguson, K. M., Berger, M. B., Mendrola, J. M., Cho, H. S., Leahy, D. J., and Lemmon, M. A. (2003) EGF activates its receptor by removing interactions that autoinhibit ectodomain dimerization, *Molecular cell 11*, 507-517.

141. Ogiso, H., Ishitani, R., Nureki, O., Fukai, S., Yamanaka, M., Kim, J. H., Saito, K., Sakamoto, A., Inoue, M., Shirouzu, M., and Yokoyama, S. (2002) Crystal structure of the complex of human epidermal growth factor and receptor extracellular domains, *Cell 110*, 775-787.

142. Lemmon, M. A., and Schlessinger, J. (2010) Cell signaling by receptor tyrosine kinases, *Cell 141*, 1117-1134.

143. Garrett, T. P., McKern, N. M., Lou, M., Elleman, T. C., Adams, T. E., Lovrecz, G. O., Zhu, H. J., Walker, F., Frenkel, M. J., Hoyne, P. A., Jorissen, R. N., Nice, E. C., Burgess, A. W., and Ward, C. W. (2002) Crystal structure of a truncated epidermal growth factor receptor extracellular domain bound to transforming growth factor alpha, *Cell 110*, 763-773.

144. Burgess, A. W., Cho, H. S., Eigenbrot, C., Ferguson, K. M., Garrett, T. P., Leahy, D. J., Lemmon, M. A., Sliwkowski, M. X., Ward, C. W., and Yokoyama, S. (2003) An open-and-shut case? Recent insights into the activation of EGF/ErbB receptors, *Molecular cell 12*, 541-552.

145. Siwak, D. R., Carey, M., Hennessy, B. T., Nguyen, C. T., McGahren Murray, M. J., Nolden, L., and Mills, G. B. (2010) Targeting the epidermal growth factor receptor in epithelial ovarian cancer: current knowledge and future challenges, *Journal of oncology 2010*, 568938.

146. Tzahar, E., Waterman, H., Chen, X., Levkowitz, G., Karunagaran, D., Lavi, S., Ratzkin, B. J., and Yarden, Y. (1996) A hierarchical network of interreceptor interactions determines signal transduction by Neu differentiation factor/neuregulin and epidermal growth factor, *Molecular and cellular biology 16*, 5276-5287.

147. Zhang, X., Gureasko, J., Shen, K., Cole, P. A., and Kuriyan, J. (2006) An allosteric mechanism for activation of the kinase domain of epidermal growth factor receptor, *Cell 125*, 1137-1149.

148. Schlessinger, J., and Lemmon, M. A. (2003) SH2 and PTB domains in tyrosine kinase signaling, *Science's STKE : signal transduction knowledge environment 2003*, RE12.

149. Pawson, T. (2004) Specificity in signal transduction: from phosphotyrosine-SH2 domain interactions to complex cellular systems, *Cell 116*, 191-203.

150. Baselga, J., and Albanell, J. (2002) Epithelial growth factor receptor interacting agents, *Hematology/oncology clinics of North America 16*, 1041-1063.

151. Levkowitz, G., Waterman, H., Ettenberg, S. A., Katz, M., Tsygankov, A. Y., Alroy, I., Lavi, S., Iwai, K., Reiss, Y., Ciechanover, A., Lipkowitz, S., and Yarden, Y. (1999) Ubiquitin ligase activity and tyrosine phosphorylation underlie suppression of growth factor signaling by c-Cbl/Sli-1, *Molecular cell 4*, 1029-1040.

152. Waterman, H., Katz, M., Rubin, C., Shtiegman, K., Lavi, S., Elson, A., Jovin, T., and Yarden, Y. (2002) A mutant EGF-receptor defective in ubiquitylation and endocytosis unveils a role for Grb2 in negative signaling, *The EMBO journal 21*, 303-313.

153. Haglund, K., and Dikic, I. (2012) The role of ubiquitylation in receptor endocytosis and endosomal sorting, *Journal of cell science 125*, 265-275.

154. Bache, K. G., Slagsvold, T., and Stenmark, H. (2004) Defective downregulation of receptor tyrosine kinases in cancer, *The EMBO journal 23*, 2707-2712.

155. Di Guglielmo, G. M., Baass, P. C., Ou, W. J., Posner, B. I., and Bergeron, J. J. (1994) Compartmentalization of SHC, GRB2 and mSOS, and hyperphosphorylation of Raf-1 by EGF but not insulin in liver parenchyma, *The EMBO journal 13*, 4269-4277.

156. von Zastrow, M., and Sorkin, A. (2007) Signaling on the endocytic pathway, *Current opinion in cell biology 19*, 436-445.

157. Miaczynska, M., Pelkmans, L., and Zerial, M. (2004) Not just a sink: endosomes in control of signal transduction, *Current opinion in cell biology 16*, 400-406.

158. Sorkin, A., and Goh, L. K. (2009) Endocytosis and intracellular trafficking of ErbBs, *Experimental cell research 315*, 683-696.

159. Normanno, N., De Luca, A., Bianco, C., Strizzi, L., Mancino, M., Maiello, M. R., Carotenuto, A., De Feo, G., Caponigro, F., and Salomon, D. S. (2006) Epidermal growth factor receptor (EGFR) signaling in cancer, *Gene 366*, 2-16.

160. Sibilia, M., Steinbach, J. P., Stingl, L., Aguzzi, A., and Wagner, E. F. (1998) A strain-independent postnatal neurodegeneration in mice lacking the EGF receptor, *The EMBO journal 17*, 719-731.

161. Miettinen, P. J., Berger, J. E., Meneses, J., Phung, Y., Pedersen, R. A., Werb, Z., and Derynck, R. (1995) Epithelial immaturity and multiorgan failure in mice lacking epidermal growth factor receptor, *Nature 376*, 337-341.

162. Downward, J., Yarden, Y., Mayes, E., Scrace, G., Totty, N., Stockwell, P., Ullrich, A., Schlessinger, J., and Waterfield, M. D. (1984) Close similarity of epidermal growth factor receptor and v-erb-B oncogene protein sequences, *Nature 307*, 521-527.

163. Libermann, T. A., Nusbaum, H. R., Razon, N., Kris, R., Lax, I., Soreq, H., Whittle, N., Waterfield, M. D., Ullrich, A., and Schlessinger, J. (1985) Amplification, enhanced expression and possible rearrangement of EGF receptor gene in primary human brain tumours of glial origin, *Nature 313*, 144-147.

164. Huang, P. H., Xu, A. M., and White, F. M. (2009) Oncogenic EGFR signaling networks in glioma, *Science signaling 2*, re6.

165. Westermark, B. (2012) Glioblastoma--a moving target, *Upsala journal of medical sciences 117*, 251-256.

166. Quick, A., Patel, D., Hadziahmetovic, M., Chakravarti, A., and Mehta, M. (2010) Current therapeutic paradigms in glioblastoma, *Reviews on recent clinical trials 5*, 14-27.

167. Raizer, J. J. (2005) HER1/EGFR tyrosine kinase inhibitors for the treatment of glioblastoma multiforme, *Journal of neuro-oncology 74*, 77-86.

168. Furnari, F. B., Fenton, T., Bachoo, R. M., Mukasa, A., Stommel, J. M., Stegh, A., Hahn, W. C., Ligon, K. L., Louis, D. N., Brennan, C., Chin, L., DePinho, R. A., and Cavenee, W. K. (2007) Malignant astrocytic glioma: genetics, biology, and paths to treatment, *Genes & development 21*, 2683-2710.

169. Stupp, R., Mason, W. P., van den Bent, M. J., Weller, M., Fisher, B., Taphoorn, M. J., Belanger, K., Brandes, A. A., Marosi, C., Bogdahn, U., Curschmann, J., Janzer, R. C., Ludwin, S. K., Gorlia, T., Allgeier, A., Lacombe, D., Cairncross, J. G., Eisenhauer, E., and Mirimanoff, R. O. (2005) Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma, *The New England journal of medicine 352*, 987-996.

170. Dunn, G. P., Rinne, M. L., Wykosky, J., Genovese, G., Quayle, S. N., Dunn, I. F., Agarwalla, P. K., Chheda, M. G., Campos, B., Wang, A., Brennan, C., Ligon, K. L., Furnari, F., Cavenee, W. K., Depinho, R. A., Chin, L., and Hahn, W. C. (2012) Emerging insights into the molecular and cellular basis of glioblastoma, *Genes & development 26*, 756-784.

171. Verhaak, R. G., Hoadley, K. A., Purdom, E., Wang, V., Qi, Y., Wilkerson, M. D., Miller, C. R., Ding, L., Golub, T., Mesirov, J. P., Alexe, G., Lawrence, M., O'Kelly, M., Tamayo, P., Weir, B. A., Gabriel, S., Winckler, W., Gupta, S., Jakkula, L., Feiler, H. S., Hodgson, J. G., James, C. D., Sarkaria, J. N., Brennan, C., Kahn, A., Spellman, P. T., Wilson, R. K., Speed, T. P., Gray, J. W., Meyerson, M., Getz, G., Perou, C. M., and Hayes, D. N. (2010) Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1, *Cancer cell 17*, 98-110.

172. Carpenter, G., and Cohen, S. (1979) Epidermal growth factor, *Annu Rev Biochem 48*, 193-216.

173. Ennis, B. W., Lippman, M. E., and Dickson, R. B. (1991) The EGF receptor system as a target for antitumor therapy, *Cancer investigation 9*, 553-562.

174. Chaffanet, M., Chauvin, C., Laine, M., Berger, F., Chedin, M., Rost, N., Nissou, M. F., and Benabid, A. L. (1992) EGF receptor amplification and expression in human brain tumours, *Eur J Cancer 28*, 11-17.

175. Hurtt, M. R., Moossy, J., Donovan-Peluso, M., and Locker, J. (1992) Amplification of epidermal growth factor receptor gene in gliomas: histopathology and prognosis, *Journal of neuropathology and experimental neurology 51*, 84-90.

176. Tohma, Y., Gratas, C., Biernat, W., Peraud, A., Fukuda, M., Yonekawa, Y., Kleihues, P., and Ohgaki, H. (1998) PTEN (MMAC1) mutations are frequent in primary glioblastomas (de novo) but not in secondary glioblastomas, *Journal of neuropathology and experimental neurology 57*, 684-689.

177. Lund-Johansen, M., Bjerkvig, R., Humphrey, P. A., Bigner, S. H., Bigner, D. D., and Laerum, O. D. (1990) Effect of epidermal growth factor on glioma cell growth, migration, and invasion in vitro, *Cancer research 50*, 6039-6044.

178. Halatsch, M. E., Gehrke, E., Borhani, F. A., Efferth, T., Werner, C., Nomikos, P., Schmidt, U., and Buchfelder, M. (2003) EGFR but not PDGFR-beta expression correlates to the antiproliferative effect of growth factor withdrawal in glioblastoma multiforme cell lines, *Anticancer research 23*, 2315-2320.

179. Shinojima, N., Tada, K., Shiraishi, S., Kamiryo, T., Kochi, M., Nakamura, H., Makino, K., Saya, H., Hirano, H., Kuratsu, J., Oka, K., Ishimaru, Y., and Ushio, Y. (2003) Prognostic value of epidermal growth factor receptor in patients with glioblastoma multiforme, *Cancer research 63*, 6962-6970.

180. Frederick, L., Wang, X. Y., Eley, G., and James, C. D. (2000) Diversity and frequency of epidermal growth factor receptor mutations in human glioblastomas, *Cancer research 60*, 1383-1387.

181. Aldape, K. D., Ballman, K., Furth, A., Buckner, J. C., Giannini, C., Burger, P. C., Scheithauer, B. W., Jenkins, R. B., and James, C. D. (2004) Immunohistochemical detection of EGFRvIII in high malignancy grade astrocytomas and evaluation of prognostic significance, *Journal of neuropathology and experimental neurology 63*, 700-707.

182. Batra, S. K., Castelino-Prabhu, S., Wikstrand, C. J., Zhu, X., Humphrey, P. A., Friedman, H. S., and Bigner, D. D. (1995) Epidermal growth factor ligand-independent, unregulated, cell-transforming potential of a naturally occurring human mutant EGFRvIII gene, *Cell growth & differentiation : the molecular biology journal of the American Association for Cancer Research 6*, 1251-1259.

183. Huang, H. S., Nagane, M., Klingbeil, C. K., Lin, H., Nishikawa, R., Ji, X. D., Huang, C. M., Gill, G. N., Wiley, H. S., and Cavenee, W. K. (1997) The enhanced tumorigenic activity of a mutant epidermal growth factor receptor common in human cancers is mediated by threshold levels of constitutive tyrosine phosphorylation and unattenuated signaling, *The Journal of biological chemistry 272*, 2927-2935.

184. Pelloski, C. E., Ballman, K. V., Furth, A. F., Zhang, L., Lin, E., Sulman, E. P., Bhat, K., McDonald, J. M., Yung, W. K., Colman, H., Woo, S. Y., Heimberger, A. B., Suki, D., Prados, M. D., Chang, S. M., Barker, F. G., 2nd, Buckner, J. C., James, C. D., and Aldape, K. (2007) Epidermal growth factor receptor variant III status defines clinically distinct subtypes of glioblastoma, *Journal of clinical oncology : official journal of the American Society of Clinical Oncology 25*, 2288-2294.

185. Moscatello, D. K., Holgado-Madruga, M., Emlet, D. R., Montgomery, R. B., and Wong, A. J. (1998) Constitutive activation of phosphatidylinositol 3-kinase by a naturally occurring mutant epidermal growth factor receptor, *The Journal of biological chemistry 273*, 200-206.

186. Feldkamp, M. M., Lala, P., Lau, N., Roncari, L., and Guha, A. (1999) Expression of activated epidermal growth factor receptors, Ras-guanosine triphosphate, and mitogen-activated protein kinase in human glioblastoma multiforme specimens, *Neurosurgery 45*, 1442-1453.

187. Antonyak, M. A., Moscatello, D. K., and Wong, A. J. (1998) Constitutive activation of c-Jun N-terminal kinase by a mutant epidermal growth factor receptor, *The Journal of biological chemistry 273*, 2817-2822.

188. Ramnarain, D. B., Park, S., Lee, D. Y., Hatanpaa, K. J., Scoggin, S. O., Otu, H., Libermann, T. A., Raisanen, J. M., Ashfaq, R., Wong, E. T., Wu, J., Elliott, R., and Habib, A. A. (2006) Differential gene expression analysis reveals generation of an autocrine loop by a mutant epidermal growth factor receptor in glioma cells, *Cancer research 66*, 867-874.

189. Nishikawa, R., Ji, X. D., Harmon, R. C., Lazar, C. S., Gill, G. N., Cavenee, W. K., and Huang, H. J. (1994) A mutant epidermal growth factor receptor common in human glioma confers enhanced tumorigenicity, *Proceedings of the National Academy of Sciences of the United States of America 91*, 7727-7731.

190. Nagane, M., Coufal, F., Lin, H., Bogler, O., Cavenee, W. K., and Huang, H. J. (1996) A common mutant epidermal growth factor receptor confers enhanced tumorigenicity on human glioblastoma cells by increasing proliferation and reducing apoptosis, *Cancer research 56*, 5079-5086.

CHAPTER 2

*A novel mechanism for the up-regulation of EGF-receptor levels in glioblastomas

Abstract

Tissue transglutaminase (tTG) is a GTP-binding protein/acyl transferase whose expression is up-regulated in glioblastoma and associated with decreased patient survival. Here we delineate a unique mechanism by which tTG contributes to the development of gliomas, by using two glioblastoma cell lines, U87 and LN229, whose growth and survival are dependent on tTG. We show that tTG significantly enhances the signaling activity and lifetimes of EGF-receptors (EGFRs) in these brain cancer cells. Moreover, over-expressing tTG in T98G glioblastoma cells that normally express low levels of tTG, caused a marked up-regulation of EGFR expression and transforming activity. We further show that tTG accentuates EGFR-signaling by blocking c-Cbl-catalyzed EGFR ubiquitylation, through the ability of tTG to bind GTP and adopt a specific conformation that enables it to interact with c-Cbl. These findings demonstrate that tTG contributes to gliomagenesis by interfering with EGFR down-regulation and thereby promoting transformation.

Introduction

Glioblastoma multiforme, also known as glioblastoma or grade IV astrocytoma, represents one of the most prevalent and aggressive forms of primary brain tumor that occurs in humans. Glioblastomas are therapeutically challenging due to the collective effects of a number of traits frequently exhibited by these types of tumors including rapid growth rates, resistance to radiation and chemotherapy, a high recurrence rate following surgical resection, and an ability to

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infiltrate surrounding normal tissue (*1*). As a result, patients with glioblastomas tend to survive only 12-17 months following their initial diagnosis, even despite having received a multimodal therapy regiment (*2*). Thus, there continues to be an over-riding need to develop additional strategies to manage this devastating form of cancer.

In an effort to identify new potential targets for therapeutic intervention, we searched for proteins whose expression is up-regulated in glioblastoma and correlated with a poor patient prognosis. One intriguing candidate that emerged is tTG, a GTP-binding protein/acyl transferase previously reported to be one of eleven metastasis-associated proteins selectively amplified in human lung and breast cancers (*3, 4*). tTG promotes the growth and survival of several different cancer cell types (*5, 6*), outcomes that are largely thought to be dependent on its acyl transferase (protein crosslinking) activity. In order to determine how tTG contributes to the development of malignant brain cancer, we used glioblastoma cell lines whose aberrant growth and survival are highly dependent upon tTG. Here we show that tTG plays an important role in the transformed properties of these cancer cells by having a major influence on EGFR protein levels and signaling activities. The ability of tTG to affect EGFR expression and function has significant implications for brain cancer given that this receptor tyrosine kinase has been shown to trigger mitogenic and survival responses in both normal astrocytes and brain tumor-derived cell lines(*7, 8*). Moreover, ectopic expression of the EGFR in normal cell types induces their transformation in a ligand-dependent manner, suggesting that increased signaling by the EGFR plays a critical role in promoting human malignancies (*9*).

At the protein level, the EGFR is over-expressed in approximately 60-90% of all glioblastomas, with the extent of EGFR expression being correlated with poor patient outcomes

(*10, 11*). Although amplification of the gene encoding the EGFR can account for the aberrant EGFR expression detected in 30-40% of primary brain tumors or brain tumor-derived cell lines (*12, 13*), additional mechanisms must be involved to account for the increased EGFR protein levels observed in those glioblastoma cases where gene amplification does not occur, as well as for the excessive and sustained EGFR-signaling that is characteristic of these brain cancers. Thus, it seemed likely that the disruption of the normal (negative) regulation of EGFRs contributes to the aberrant EGFR-signaling capabilities exhibited in at least some glioblastomas. Indeed, it is through the regulation of EGFR degradation where tTG appears to exert a major influence, because we show that it affects the ability of c-Cbl, an E3 ubiquitin ligase, to target the EGFR for lysosomal degradation. This involves the ability of tTG, when bound to GTP and having adopted a specific GTP-induced conformational state, to associate with c-Cbl and block the c-Cbl-catalyzed ubiquitylation and degradation of EGFRs, thereby significantly enhancing and extending EGFR-signaling activities.

Results

tTG is over-expressed in human brain tumors

tTG functions both as a GTPase and acyl transferase whose expression and activation have been shown to be up-regulated in several different types of human cancer including breast, ovarian, and pancreatic cancer (*14-16*). In addition, tTG has been demonstrated to play an important role in the growth, survival, migration and invasive activity of aggressive cancer cells (*17*). These findings, coupled with the fact that tTG was identified as a down-stream signaling partner of the EGFR, promoting the transformed characteristics of human breast cancer SKBR3 cells (*6*), as well as having an essential function in the EGF-stimulated migration and invasion of

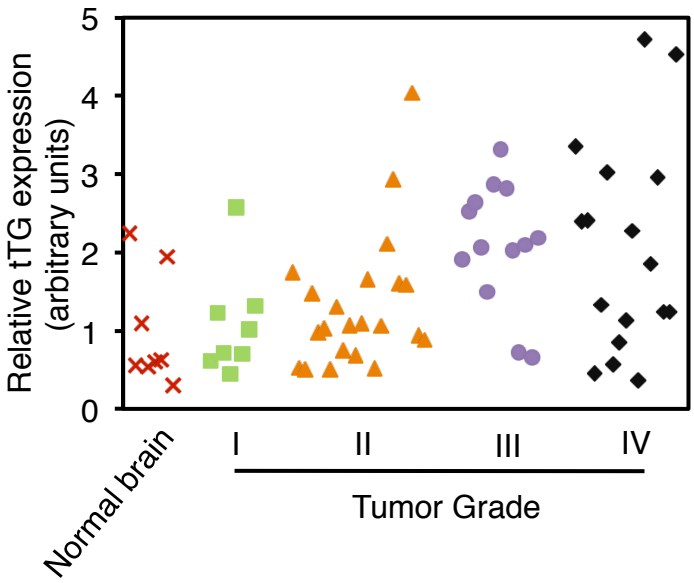
different cancer cells (*18, 19*), made it attractive to consider that tTG plays an important role in the development of brain cancer.

We began by examining tTG expression levels in human primary brain tumors. A tissue-array consisting of normal brain tissues and astrocytomas ranging from grade I to grade IV (grade IV astrocytomas are also referred to as glioblastomas) was immuno-stained for tTG. We found that, like the case for the normal human astrocyte (NHA) controls, tTG expression in normal brain is generally low (Figures 2.1A, 2.1B, and 2.1D, top panel). Similarly, tTG is often expressed at relatively low levels in the lower grade (grades I and II) astrocytomas (Figure 2.1C, left panel). However, in the more advanced/aggressive brain tumors (i.e. grades III and IV astrocytomas), tTG expression was frequently up-regulated. For example, greater than 80% of the grade III astrocytomas and nearly 60% of the grade IV astrocytomas showed increased tTG expression (Figure 2.1A). Figure 2.1B shows a representative comparison of tTG expression in a normal brain sample versus a grade IV astrocytoma. We then took our analysis one step further by comparing tTG transcript levels in the four sub-types of glioblastomas that have been identified according to their unique molecular signatures (*20*) using The Cancer Genome Atlas (TCGA). Figure 2.1E shows that tTG transcript levels are increased in the mesenchymal (~45%) and classical (~16%) tumor sub-types.

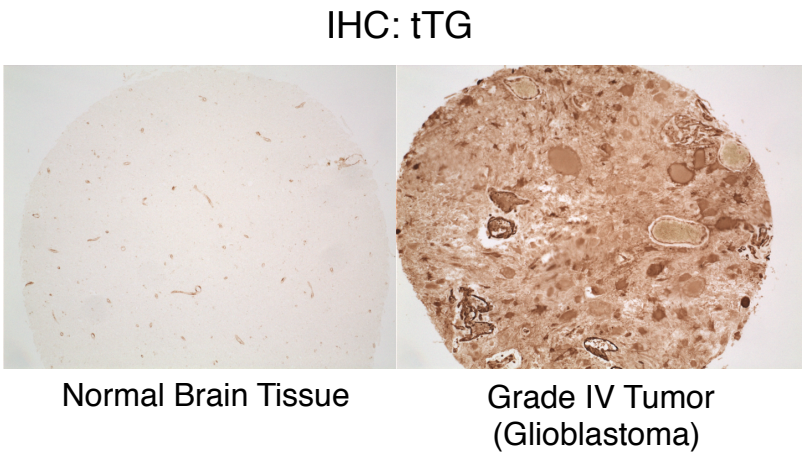
Overall, the trend we observed for tTG expression matched that for EGFR expression and phosphorylation (Figure 2.1C, middle and right panels). While we were not able to draw a strict correlation between tTG expression and the survival of glioblastoma patients (Figure 2.1F, left panel), we did find a correlation based on tumor grade, similar to what has been observed for the EGFR (*11, 21*). Individuals with gliomas of various grades whose tumors showed a 2-fold or greater increase in tTG expression had an ~25% reduction in their expected lifespan, whereas

Figure 2.1. tTG expression is up-regulated in high grade human brain tumors and correlates with poor patient outcomes.
(A-C) Tissue arrays of human primary brain tumors of increasing grades and normal brain samples were subjected to immunohistochemical analysis using tTG, EGFR and phospho-EGFR antibodies.
(A) Expression levels of tTG detected in the tissue array. Each symbol on the chart represents an individual normal brain or tumor sample as indicated.
(B) Representative images of a normal brain sample and a grade IV tumor (glioblastoma) stained for tTG. Magnification is ×10.
(C) The expression levels of tTG (left) and the EGFR (middle), and the levels of EGFR phosphorylation (right) shown represent their enhanced expression in brain tumor tissue relative to their expression in normal brain tissue (which was set to 1).
(D) Cell extracts from serum-starved normal human astrocytes (NHAs) and various brain tumor cell lines, were immunoblotted (IB) with tTG and actin antibodies (top two panels). The extracts were also assayed for their enzymatic transamidation activity, as read-out by the incorporation of BPA into lysate proteins (bottom panel).
(E) The percentage of glioblastomas exhibiting increased tTG transcript levels was determined and then plotted by sub-type.
(F) Kaplan-Meier Survival Plots for glioblastoma (GBM) patients (left panel) and all glioma patients (right panel) with differential tTG expression levels. Data cited from REMBRANDT, National Cancer Institute, accessed 2013, March 18. Downreg, downregulation; Upreg, upregulation.

A


Relative tTG expression
(arbitrary units)
5
4
3
2
1
0
Normal brain
I
II
III
IV
Tumor Grade

B


IHC: tTG
Normal Brain Tissue
Grade IV Tumor
(Glioblastoma)

C

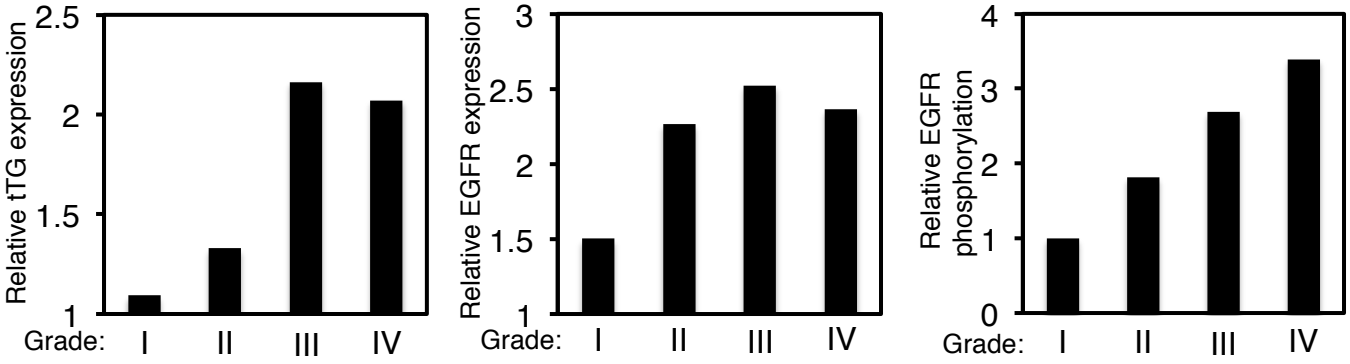
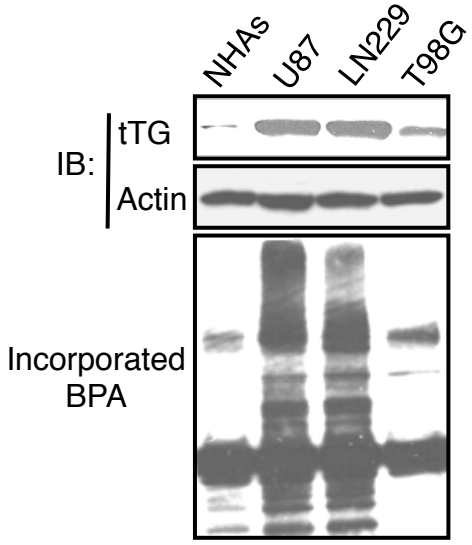
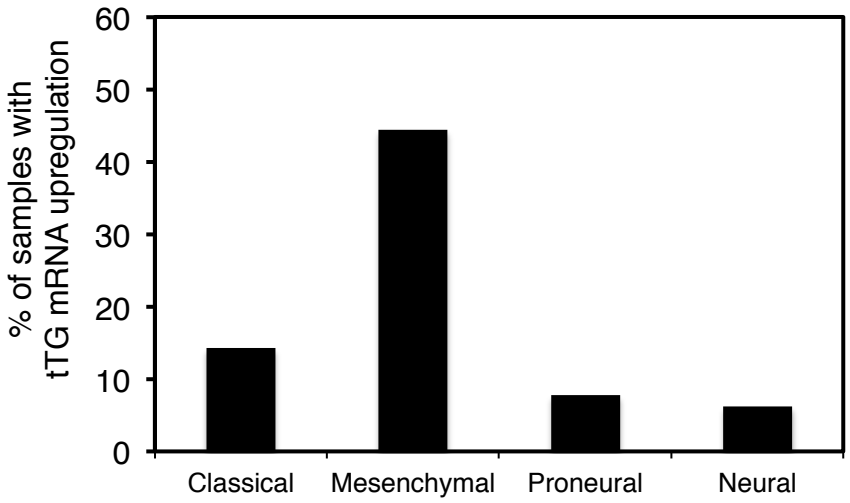

Relative tTG expression
2.5
2
1.5
1
Grade: I II III IV
Relative EGFR expression
3
2.5
2
1.5
1
Grade: I II III IV
Relative EGFR phosphorylation
4
3
2
1
0
Grade: I II III IV

Figure 2.1 continued

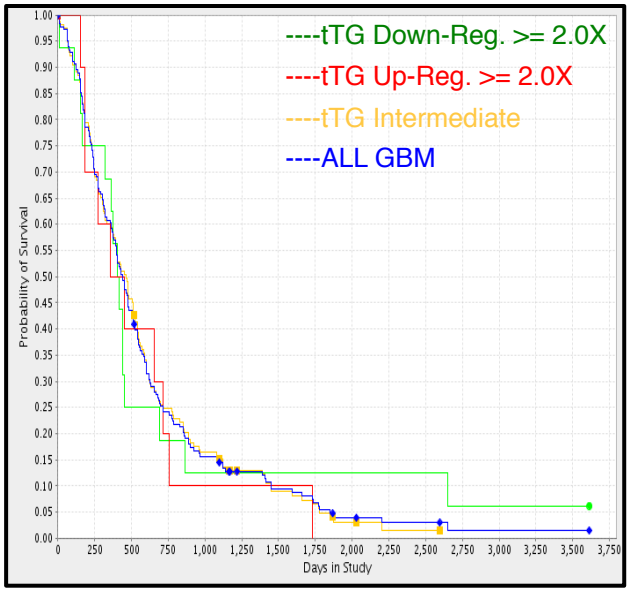
D

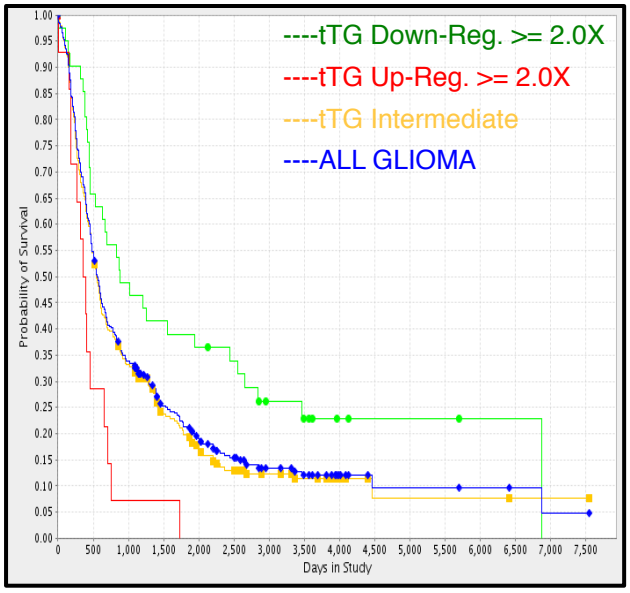


E



F





those individuals with a 2-fold or greater decrease in tTG expression benefited by an ~20% increase in their survival (Figure 2.1F, right panel).

tTG is essential for the transformed properties of glioblastoma cell lines

In order to learn more about the potential role played by tTG in brain cancer, we set out to identify human glioblastoma cell lines that exhibited tTG-dependent transformed phenotypes. Three commonly used human glioblastoma cell lines, U87, LN229, and T98G cells, were examined for tTG expression and acyl transferase activity. As shown in Figure 2.1D, top panel, U87 and LN229 cells exhibited significantly higher levels of tTG expression compared to NHAs, while T98G cells exhibited only a modest increase in tTG levels. The same was true when comparing the enzymatic transamidation activity catalyzed by tTG in lysates prepared from these different cell lines, as read-out by assaying the incorporation of biotinylated pentylamine (BPA) into their lysate proteins (Figure 2.1D, bottom panel) (*22*).

We next asked whether the expression of tTG in these glioblastoma cells contributed to their ability to exhibit transformed phenotypes. The anchorage-independent growth of U87, LN229, and T98G cells was assayed under conditions where tTG activity was blocked using two distinct tTG inhibitors, monodansyl cadaverine (MDC) and Z-Don, or after tTG expression was knocked-down by siRNA (Figures 2.2A and 2.2B). Figure 2.2C shows that the ability of both U87 and LN229 cells to form colonies in soft agar was sensitive to MDC and Z-Don, with the number of colonies formed by each of these cell lines being reduced by at least 60% upon treatment with the inhibitors. Likewise, the knock-down of tTG expression in U87 and LN229 cells using two different siRNAs (Figure 2.2D, insets) inhibited colony formation (Figure 2.2D). In contrast, the anchorage-independent growth of T98G glioblastoma cells was not dependent on

Figure 2.2. tTG is essential for the transformed characteristics of U87 and LN229 glioblastoma cells.

(A) Cell extracts from serum-starved U87, LN229 and T98G cells treated without or with MDC or Z-Don for 1 day were immunoblotted (IB) with tTG and actin antibodies (bottom two panels), as well as assayed for their enzymatic transamidation activity (top panel).
(B) Cell extracts from U87, LN229 and T98G cells expressing control or tTG siRNAs were immunoblotted (IB) with tTG and actin antibodies (bottom two panels), or assayed for their enzymatic transamidation activity (top panel).
(C) Soft agar colony formation assays were performed on U87, LN229 and T98G cells treated without or with MDC or Z-Don. After 10 days of growth, the colonies that formed were counted.
(D) U87, LN229, and T98G cells transfected with control or tTG siRNAs were either lysed and immunoblotted (IB) with tTG and actin antibodies (insets), or subjected to soft agar colony formation assays. After 10 days of growth, the colonies that formed were counted.
(E) Apoptotic assays were performed on U87, LN229 and T98G cells cultured in serum-free medium supplemented without or with MDC, and without or with doxorubicin (Dox), for 1 day. Apoptotic cells were identified by the presence of condensed or blebbed nuclei.
(C-E) Data are represented as mean ± SEM.

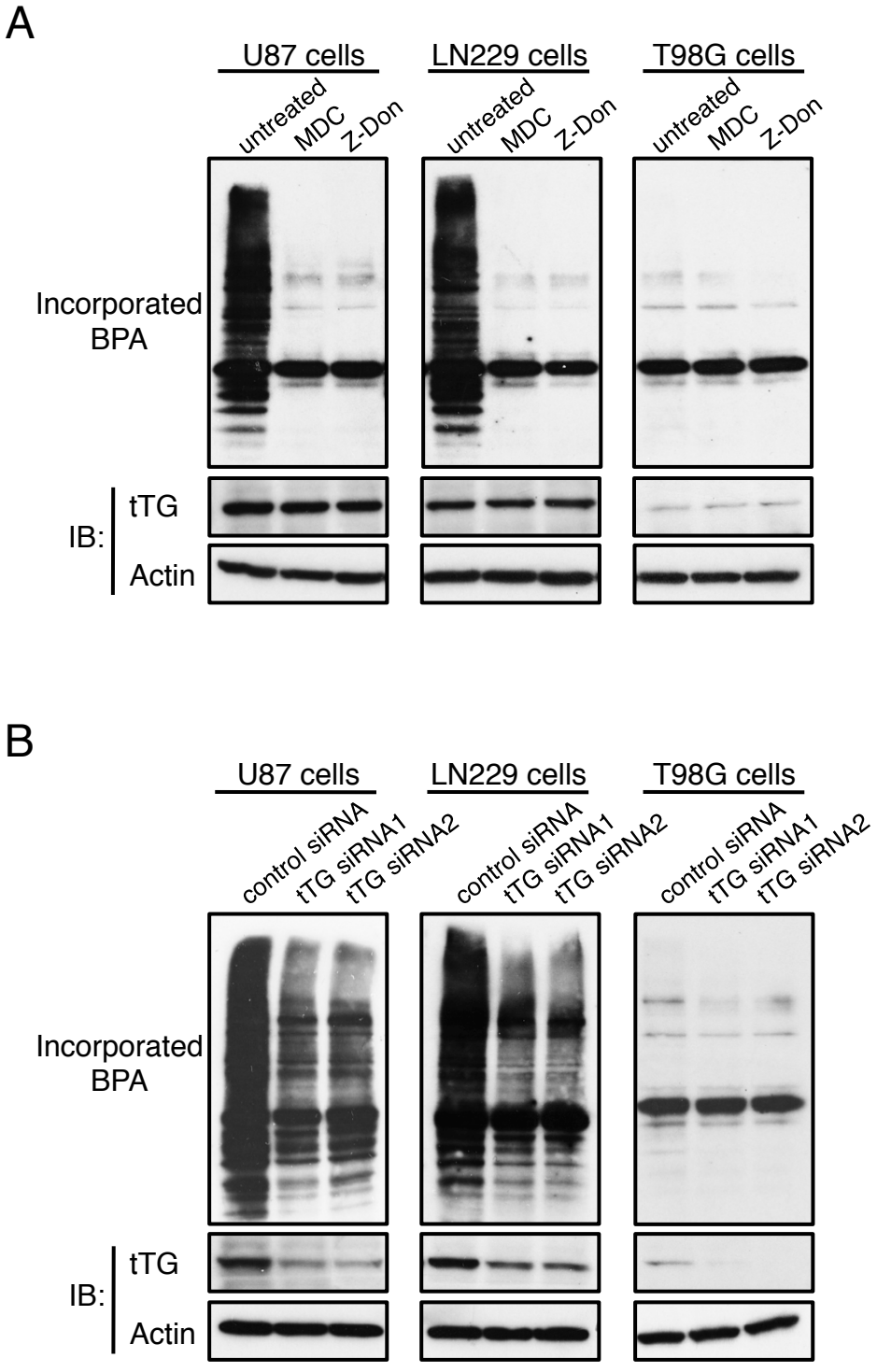
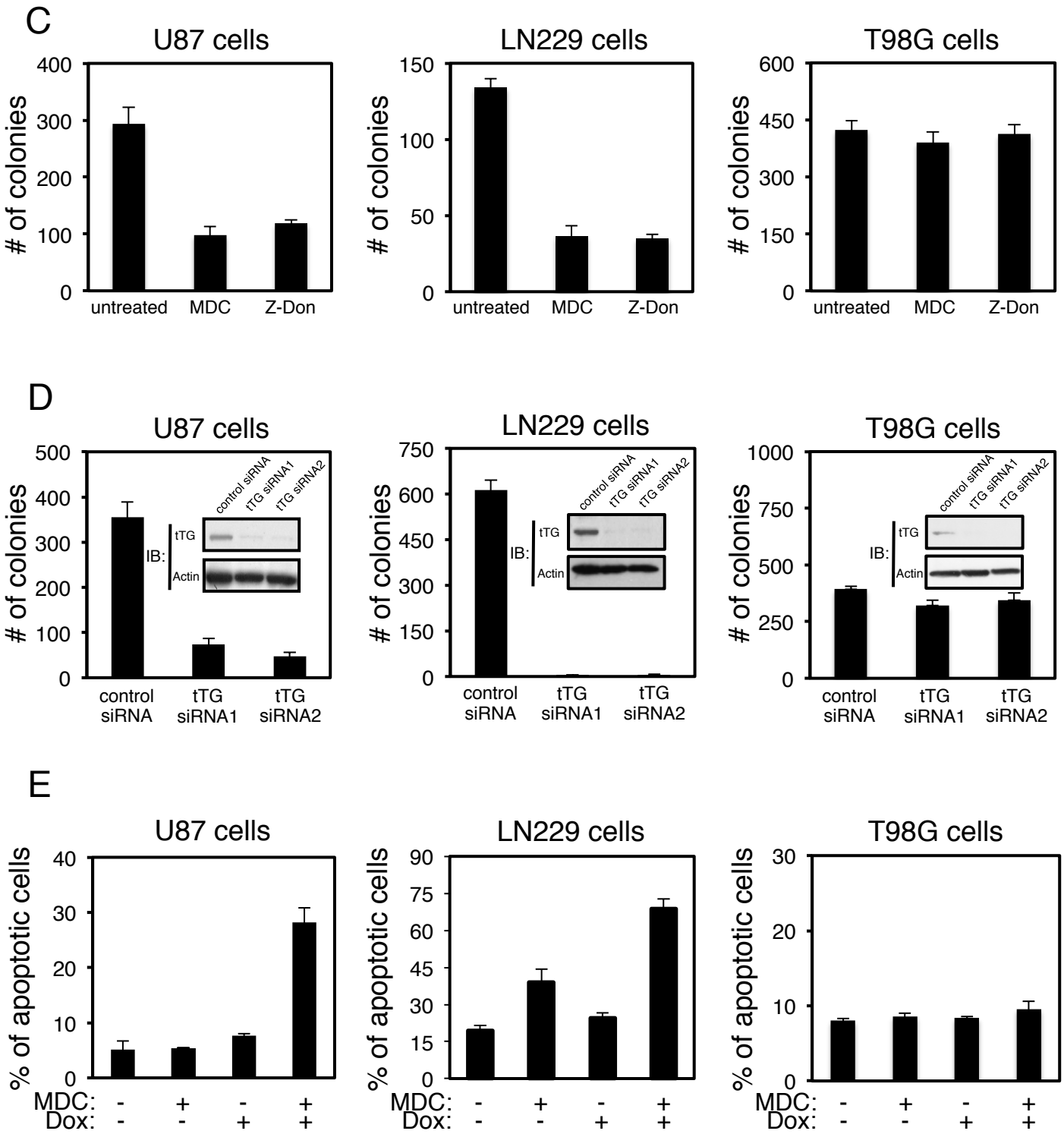

A
U87 cells
LN229 cells
T98G cells
untreated
MDC
Z-Don
Incorporated BPA
IB:
tTG
Actin
B
U87 cells
LN229 cells
T98G cells
control siRNA
tTG siRNA1
tTG siRNA2
Incorporated BPA
IB:
tTG
Actin

Figure 2.2 continued



tTG, as it was insensitive to tTG inhibitors MDC and Z-Don, as well as to siRNAs targeting tTG (Figures 2.2C and 2.2D).

It was previously shown that these brain cancer cells are resistant to chemotherapy (*23-25*), and indeed challenging the different glioblastoma cell lines with doses of doxorubicin that potently killed other types of human cancer cells (i.e. SKBR3 breast cancer cells) (*22*), induced only modest increases in cell death beyond that normally observed in the absence of any treatment (Figure 2.2E). When assaying the survival of these glioblastoma cell lines following their exposure to various combinations of MDC and doxorubicin, we found that MDC treatment alone caused little or no increase in their rates of apoptosis (Figure 2.2E). However, when the cells were treated with both MDC and doxorubicin, an ~2-3 fold increase in apoptosis occurred in U87 and LN229 cells compared to what was observed with doxorubicin alone (Figure 2.2E, left and middle panels), whereas, treatment of T98G cells with the combination of MDC and doxorubicin showed essentially no effect (Figure 2.2E, right panel). Together, these findings demonstrate that the over-expression of tTG that occurs in highly aggressive human brain tumors and glioblastoma cell lines (U87 and LN229) strongly contributes to their aberrant growth and chemo-resistance.

tTG regulates EGFR levels and the extent of EGFR-signaling activities in brain tumor cells

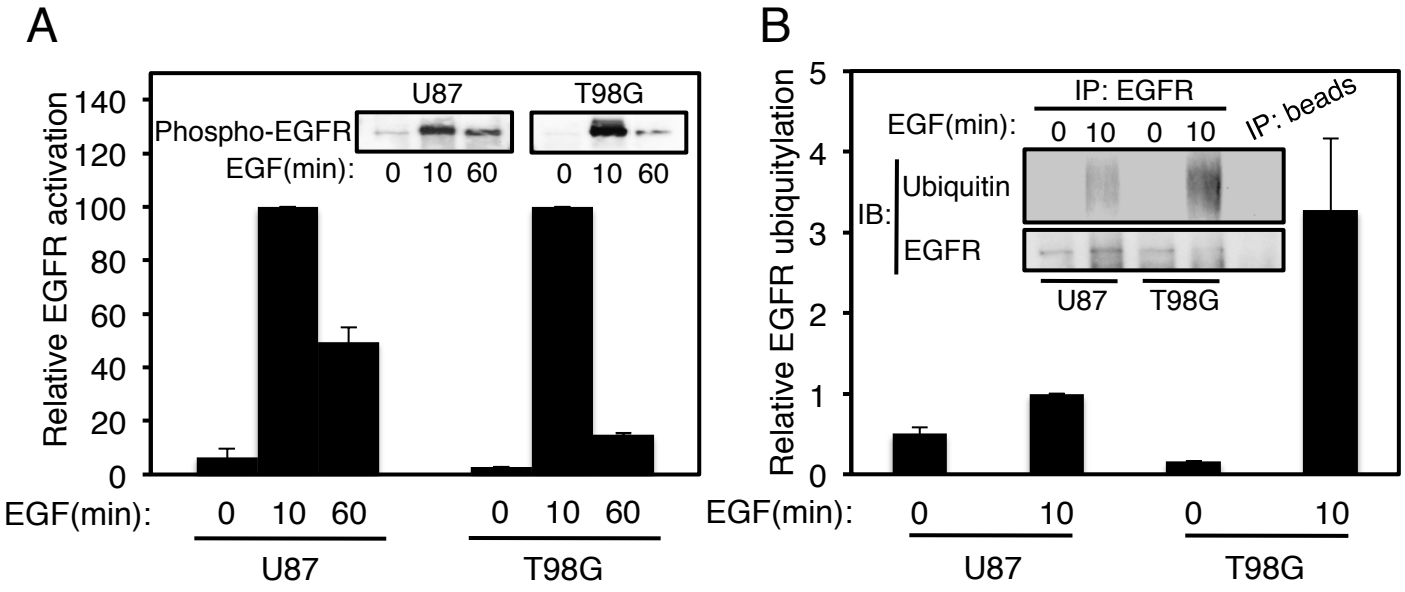
Both tTG and EGFR expression levels are frequently up-regulated in high grade brain tumors. Thus, we examined whether EGFR-signaling might be enhanced and/or extended in a glioblastoma cell line that showed relatively high tTG expression and whose transformed properties were dependent upon tTG (U87 cells), compared to a cell line that expressed relatively low levels of tTG and was not dependent upon it for transformation (T98G cells). Indeed, we

found that the signaling lifetimes of activated EGFRs in U87 cells were extended, compared to the case for T98G cells (Figure 2.3A). This appeared to be preceded by a significantly greater extent of EGFR ubiquitylation in T98G cells compared to U87 cells (Figure 2.3B).

We then set out to establish that tTG is responsible for enhancing EGFR-signaling in glioblastoma. First, we took advantage of the fact that T98G glioblastoma cells have relatively low levels of tTG expression and are not dependent upon tTG for their survival or transformed growth properties. Interestingly, these brain cancer cells also showed virtually no dependence on EGFR-signaling for their transformed characteristics, as indicated by their insensitivity to the EGFR tyrosine kinase inhibitor AG1487 (Figure 2.3C, compare the first and second histograms). We then generated T98G cells that stably expressed either vector-alone or a Myc-tagged form of tTG (Figure 2.3D, top two panels). As expected, lysates from the cells over-expressing Myc-tagged tTG exhibited significantly more transamidation activity than lysates from cells expressing vector-alone (Figure 2.3D, bottom panel). While T98G cells that expressed just the vector-alone were capable of forming colonies in soft agar, cells that ectopically expressed tTG showed a significant increase (i.e. ~2-fold) in their anchorage-independent growth (Figure 2.3C, compare the first and third bars). Moreover, upon ectopically expressing tTG in T98G cells, the EGFR protein levels increased by 3- to 4-fold (Figure 2.3E, top panel, compare the first and third lanes). This increase in EGFR levels was off-set upon treatment of the cells with the tTG inhibitor MDC (Figure 2.3E, compare the third and fourth lanes), thereby directly demonstrating that tTG influences EGFR expression. We then asked whether the ability of tTG to potentiate EGFR levels in T98G transfectants was important for the enhanced transforming capabilities exhibited by the T98G cells stably over-expressing tTG. As shown in Figure 2.3C, the enhanced colony formation exhibited by T98G cells upon the ectopic expression of tTG was nearly

Figure 2.3. EGFR signaling is potentiated in U87 glioblastoma cells.

(A) Extracts from U87 and T98G cells that had been cultured in serum-free medium and then further stimulated without or with EGF for increasing lengths of time, were immunoblotted (IB) using a phospho-EGFR antibody (insets). The relative activity of the EGFR in each cell line was plotted (the EGFR activity detected at 10 min of EGF stimulation was set as the maximal activity for each cell line).
(B) Immunoprecipitations with an EGFR antibody (IP: EGFR) were performed on the extracts from U87 and T98G cells that had been cultured in serum-free medium and then further stimulated without or with EGF for 10 min. The resulting immuno-complexes were immunoblotted (IB) with EGFR and ubiquitin antibodies, and the relative ubiquitylation levels of the EGFR for each sample were plotted (the level of EGFR ubiquitylation detected in U87 cells stimulated with EGF for 10 min was set as 1).
(C) Soft agar colony formation assays were performed on the T98G stable cell lines treated without or with AG1478.
(A-C) Data are represented as mean ± SEM.
(D) Extracts from T98G cells stably expressing the vector-alone or a Myc-tagged wild-type tTG (tTG WT) were immunoblotted (IB) with Myc, tTG, and actin antibodies (top three panels), as well as assayed for their enzymatic transamidation activity, as read-out by the incorporation of BPA into lysate proteins (bottom panel).
(E) Extracts from the T98G stable cell lines cultured in serum-free medium supplemented without or with MDC were immunoblotted (IB) with EGFR, tTG and actin antibodies.


A
Relative EGFR activation
140
120
100
80
60
40
20
0
U87
T98G
Phospho-EGFR
EGF(min): 0 10 60 0 10 60
EGF(min): 0 10 60 0 10 60
U87
T98G
B
Relative EGFR ubiquitylation
5
4
3
2
1
0
IP: EGFR
EGF(min): 0 10 0 10
IP: beads
IB: Ubiquitin
EGFR
U87
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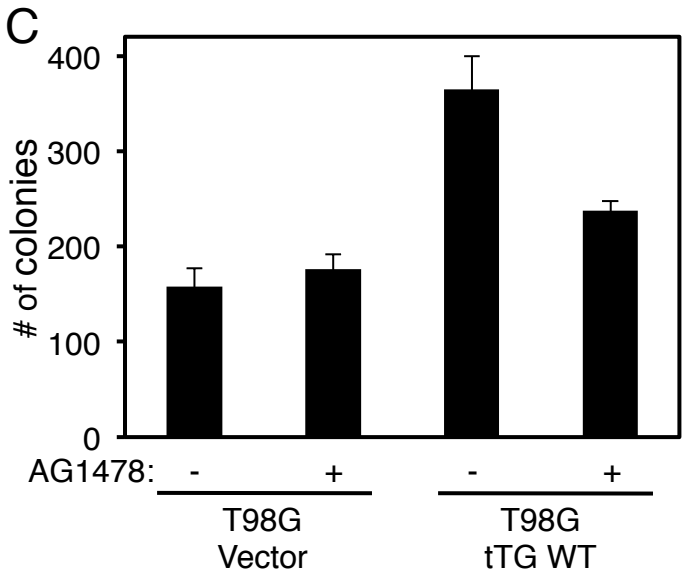
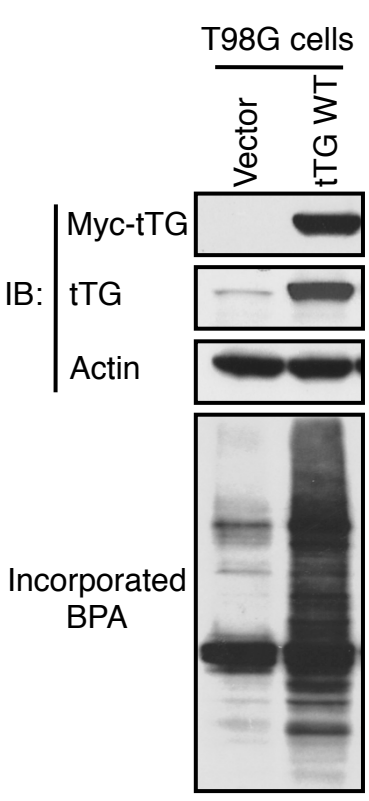
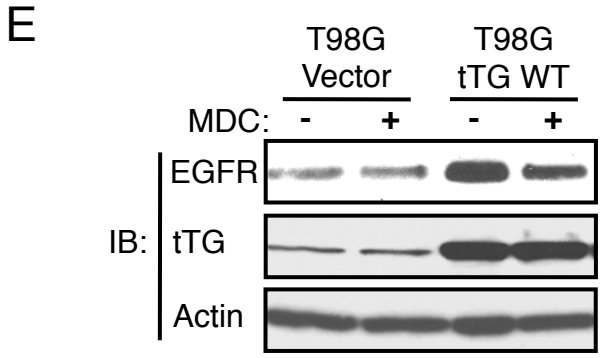
EGF(min): 0 10 0 10
U87
T98G


C
# of colonies
400
300
200
100
0
AG1478: - + - +
T98G Vector
T98G tTG WT


D
T98G cells
Vector
tTG WT
IB: Myc-tTG
tTG
Actin
Incorporated BPA


E
T98G Vector
T98G tTG WT
MDC: - + - +
IB: EGFR
tTG
Actin

completely eliminated by AG1478 (compare the third and fourth histograms), indicating that the enhancement in transformation accompanying over-expression of tTG was dependent upon EGFR tyrosine kinase activity.

We then made use of the U87 and LN229 glioblastoma cell lines to further establish that tTG mediates the up-regulation of EGFR expression and signaling. Figure 2.4A (top two panels) shows that treatment of both U87 and LN229 cells with the tTG inhibitor MDC consistently reduced the basal levels of EGFR expression and activation, as read-out using an anti-pan-EGFR antibody and an anti-phospho-EGFR antibody that detects EGFR auto-phosphorylation, respectively. Likewise, MDC treatment markedly reduced the amount of EGFR-stimulated phosphorylation of c-Jun, ERK and Akt in both cell lines (Figure 2.4A, third, fifth and seventh panels), signaling outcomes occurring downstream from the EGFR that are frequently observed in primary brain tumors and brain tumor-derived cell lines, including U87 cells (Figure 2.4B) (*26-28*). To further confirm a role for tTG in regulating EGFR expression levels, we examined whether a decrease in the cellular steady-state levels of the EGFR accompanied siRNA-mediated knock-downs of tTG. Indeed, the siRNA-induced reduction in tTG levels by ~80% in U87 cells and ~50% in LN229 cells (Figure 2.4C, second panels from the top), resulted in corresponding decreases in EGFR expression (Figure 2.4C, top panels).

We next examined whether tTG influenced the extent of EGFR-signaling activities in these glioblastoma cells. Serum-starved cultures of U87 cells were treated with EGF for increasing lengths of time, in the presence or absence of MDC. Figure 2.4D shows that a maximal activation of EGFR tyrosine kinase activity occurred within 10 minutes of growth factor treatment (top panel). EGF stimulation of U87 cells pretreated with MDC showed a marked reduction in the magnitude of EGFR activation. Cell surface biotin-labeling experiments

Figure 2.4. tTG regulates EGFR levels and signaling activity in glioblastoma cells.

(A) Extracts from U87 and LN229 cells cultured in serum-free medium supplemented without or with MDC for 1 day were immunoblotted (IB) using the indicated antibodies. The fold changes in EGFR levels, as determined using ImageJ, are highlighted.
(B) The basal c-Jun, ERK and Akt activities in U87 cells are dependent on the EGFR. Cell extracts from serum-starved U87 cells treated without or with AG1478 for 1 day were immunoblotted (IB) using phospho-specific EGFR, c-Jun, ERK and Akt antibodies, as well as with tTG and actin antibodies.
(C) Extracts from U87 cells and LN229 cells expressing control or tTG siRNAs were immunoblotted (IB) using EGFR, tTG, and actin antibodies. The fold changes in EGFR levels, as determined using ImageJ, are highlighted.
(D) Extracts from U87 cells that had been cultured in serum-free medium and then treated without or with MDC for 30 min before being further stimulated with EGF for increasing lengths of time, were immunoblotted (IB) using the indicated antibodies.
(E) U87 cells treated without or with MDC for 1 day were incubated with a cell impermeable biotinylation reagent to label surface proteins and then lysed. Immunoprecipitations with an EGFR antibody (IP: EGFR) were performed on the cell extracts. The resulting immuno-complexes were first blotted with Streptavidin-HRP to detect the amount of cell surface EGFR and then were re-probed with an EGFR antibody to confirm that the EGFR was immunoprecipitated. A small sample of the cell extracts (WCLs) was also immunoblotted with the indicated antibodies.
(F) EGF-stimulated activation of the EGFR in LN229 glioblastoma cells is attenuated by MDC treatment. Cell extracts from LN229 cells that had been cultured in serum-free medium and then were treated without or with MDC for 30 minutes before being further stimulated with EGF for the indicated lengths of time, were immunoblotted (IB) using the phospho-EGFR, as well as with tTG and actin antibodies.
(G) EGFRvIII expression levels are not influenced by tTG. U87 cells stably expressing either the vector-alone or EGFRvIII were cultured in serum free medium with or without MDC for 1 day before being lysed. The cell extracts were then immunoblotted with EGFR, tTG and actin antibodies. Note that MDC treatment reduces the levels of the endogenous wild-type EGFR, but not that of the ectopically expressed EGFRvIII.

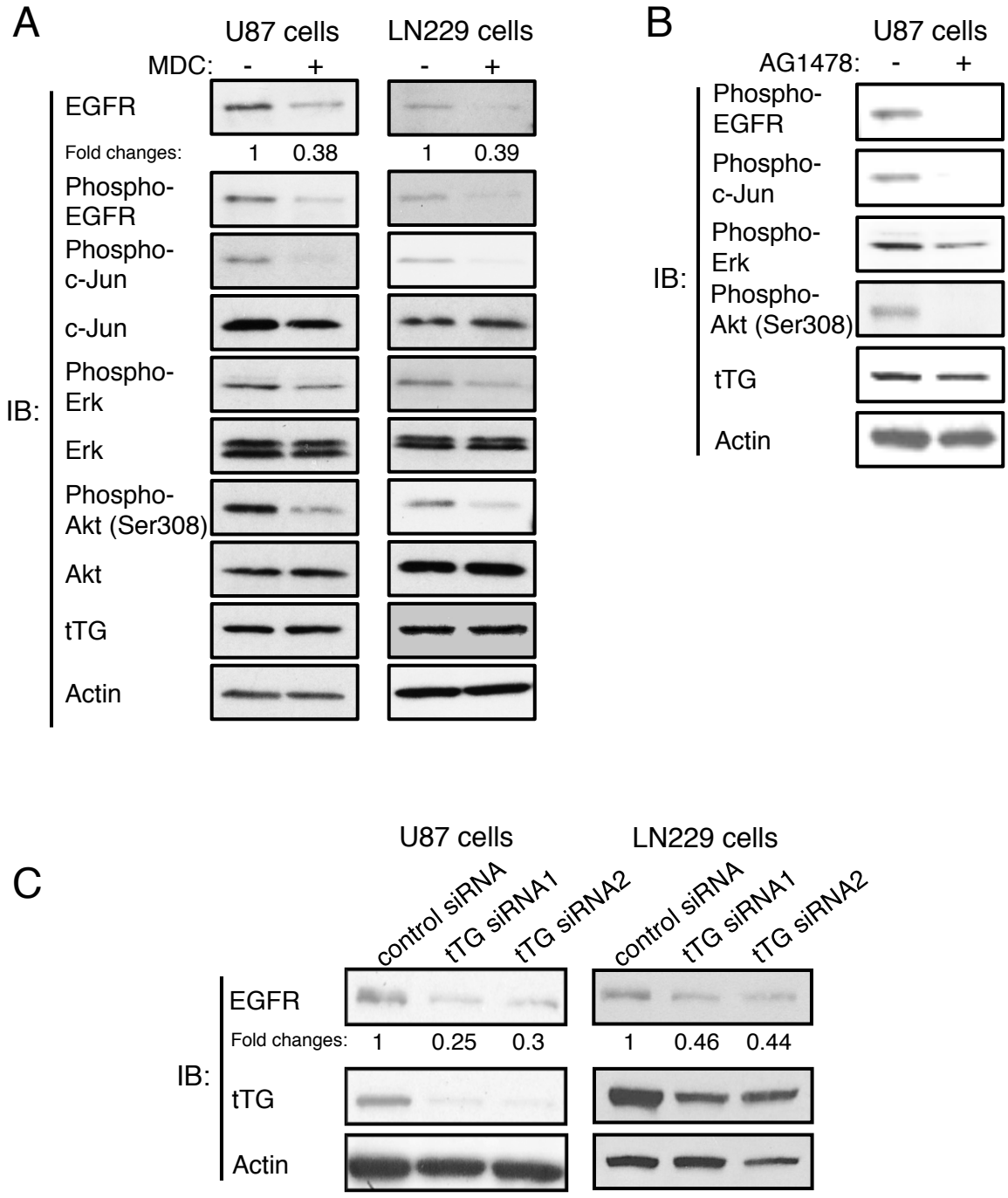
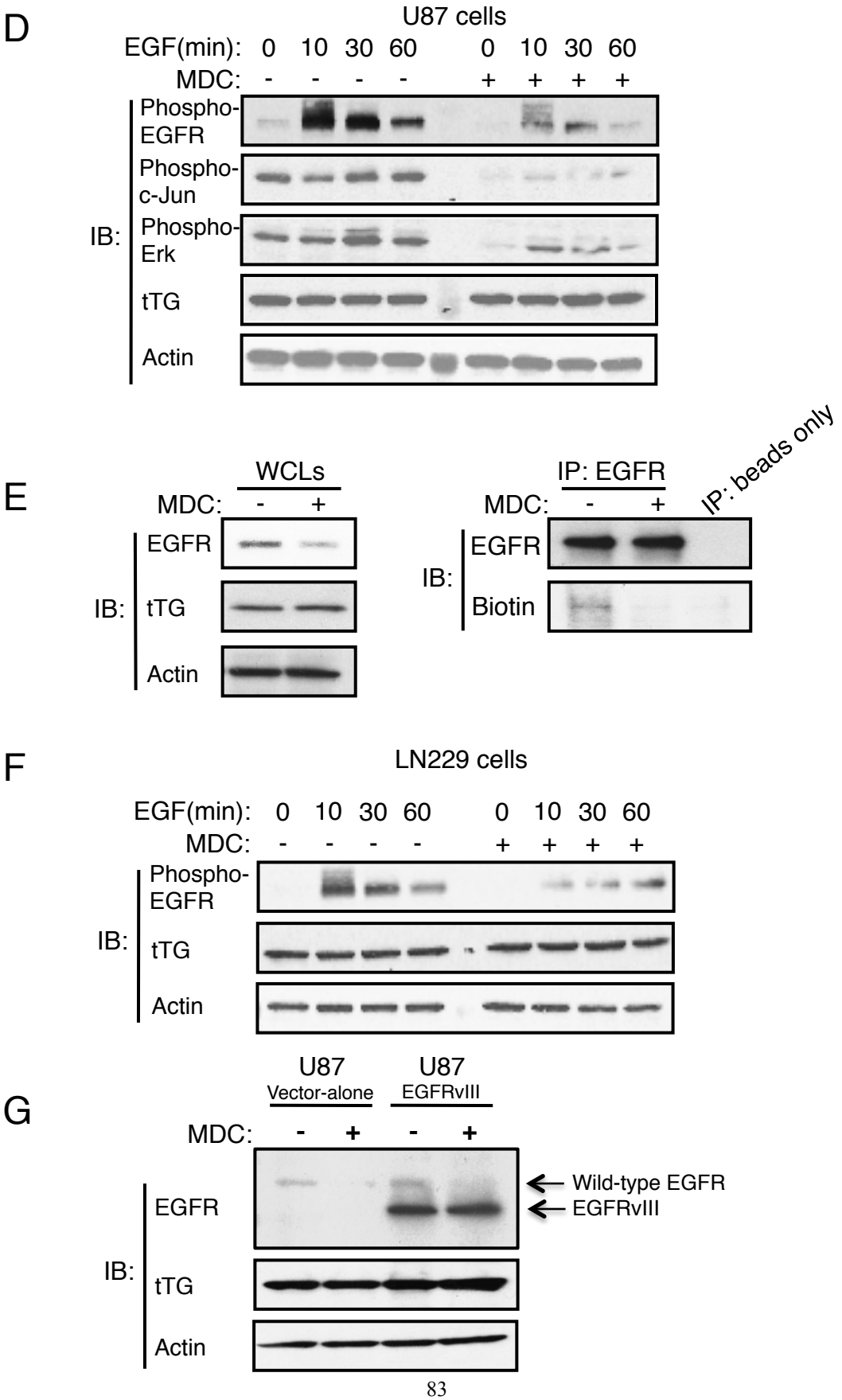

A
U87 cells
LN229 cells
MDC: - + - +
EGFR
Fold changes: 1 0.38 1 0.39
Phospho-EGFR
Phospho-c-Jun
c-Jun
Phospho-Erk
Erk
Phospho-Akt (Ser308)
Akt
tTG
Actin
IB:
B
U87 cells
AG1478: - +
Phospho-EGFR
Phospho-c-Jun
Phospho-Erk
Phospho-Akt (Ser308)
tTG
Actin
IB:
C
U87 cells
LN229 cells
control siRNA
tTG siRNA1
tTG siRNA2
control siRNA
tTG siRNA1
tTG siRNA2
EGFR
Fold changes: 1 0.25 0.3 1 0.46 0.44
tTG
Actin
IB:

Figure 2.4 continued



performed on the cells showed that this was due to reductions in the EGFR levels at the plasma membrane (Figure 2.4E). Moreover, the phosphorylation of c-Jun (Figure 2.4D, second panel from the top) and ERK (Figure 2.4D, third panel from the top) was significantly reduced in U87 cells pretreated with MDC. While each of these signaling events showed little dependence on EGF stimulation, the basal activities of both were dependent on EGFR activity as indicated by the inhibition caused by the specific EGFR tyrosine kinase inhibitor AG1478 (Figure 2.4B). EGFR activation was also compromised in LN229 glioblastoma cells treated with the tTG inhibitor MDC (Figure 2.4F).

We also examined whether tTG could influence the levels of the EGFR variant type III (EGFRvIII), a commonly occurring and highly oncogenic mutant form of the EGFR that is defective in its down-regulation (*29*). Figure 2.4G shows that ectopic EGFRvIII expression in U87 cells was not affected by MDC treatment (compare the first and second lanes to the third and fourth lanes). This suggests that tTG is not important for extending the lifetime of this mutant EGFR, most likely because unlike the wild-type EGFR, this truncated EGFR variant has an inherent insensitivity to the degradative actions of c-Cbl (*30*) (also see below).

tTG blocks ubiquitylation of the EGFR

Taken together, the results presented in Figures 2.3 and 2.4, as well as those shown in Figure 2.5A, left panel, suggest that the actions of tTG are directed at enhancing the stability of wild-type EGFRs. The ubiquitylation-mediated down-regulation of the EGFR, as catalyzed by the E3 ubiquitin ligase c-Cbl, plays an essential role in terminating EGFR-signaling activities by targeting the receptor for degradation in the lysosomes (*31, 32*). Figure 2.5A, right panel, shows that following treatment of U87 cells with MDC or Z-Don, the amount of detectable

Figure 2.5. tTG influences the ubiquitylation of the EGFR by associating with E3 ubiquitin ligase c-Cbl.

(A) Immunoprecipitations with an EGFR antibody (IP:EGFR) were performed on the extracts from U87 cells that were cultured in serum-free medium supplemented without or with MDC or Z-Don for 1 day. The resulting immuno-complexes as well as samples of the whole cell lysates (WCLs) were immunoblotted (IB) with EGFR, ubiquitin, tTG and actin antibodies.
(B) Cell extracts from U87 cells treated without or with MDC or Z-Don for 1 day were immunoblotted (IB) with ubiquitin and actin antibodies.
(C) Immunoprecipitations with an EGFR antibody (IP: EGFR) were performed on the extracts from U87 cells expressing control or tTG siRNAs. The resulting immuno-complexes were then immunoblotted (IB) with EGFR and ubiquitin antibodies. The fold changes in ubiquitinylated EGFR, as determined using ImageJ, are highlighted.
(D) Immunoprecipitations with an EGFR antibody (IP: EGFR) were performed on the extracts from LN229 cells cultured in serum-free medium supplemented without or with MDC, for 1 day. The resulting immuno-complexes were then immunoblotted (IB) with EGFR and ubiquitin antibodies.
(E) Immunoprecipitations with an HA antibody (IP: HA-c-Cbl) were performed on extracts from U87 cells transfected with HA-tagged c-Cbl (HA-c-Cbl) and cultured in serum-free medium supplemented without or with MDC for 1 day. The resulting immuno-complexes, as well as the whole cell extracts collected (WCLs), were immunoblotted (IB) with tTG and HA antibodies.
(F) List of the various tTG constructs used in this study including: the name of each construct, the sites of the mutations, and their functional consequences on nucleotide binding and enzymatic transamidation activity are indicated.
(G) Immunoprecipitations with an HA antibody (IP: HA-c-Cbl) were performed on extracts from U87 cells transfected with HA-tagged c-Cbl and either Myc-tagged wild-type tTG (Myc-tTG WT) or one of the indicated Myc-tagged mutant forms of tTG listed in D. The resulting immuno-complexes, as well as the whole cell extracts collected (WCLs), were immunoblotted (IB) with HA, Myc, and actin antibodies.
(H) Purified, recombinant His-tagged, wild-type (WT) tTG, or one of the mutant forms of tTG, were incubated alone or together with an equal amount of recombinant GST-tagged c-Cbl. The resulting protein complexes were precipitated with GST beads and then immunoblotted with tTG and c-Cbl antibodies (Pull-down: GST). Each form of His-tagged tTG used in the pull-down assay was also immunoblotted with a tTG antibody to confirm that equal amounts of recombinant tTG were used in the experiment (Input).

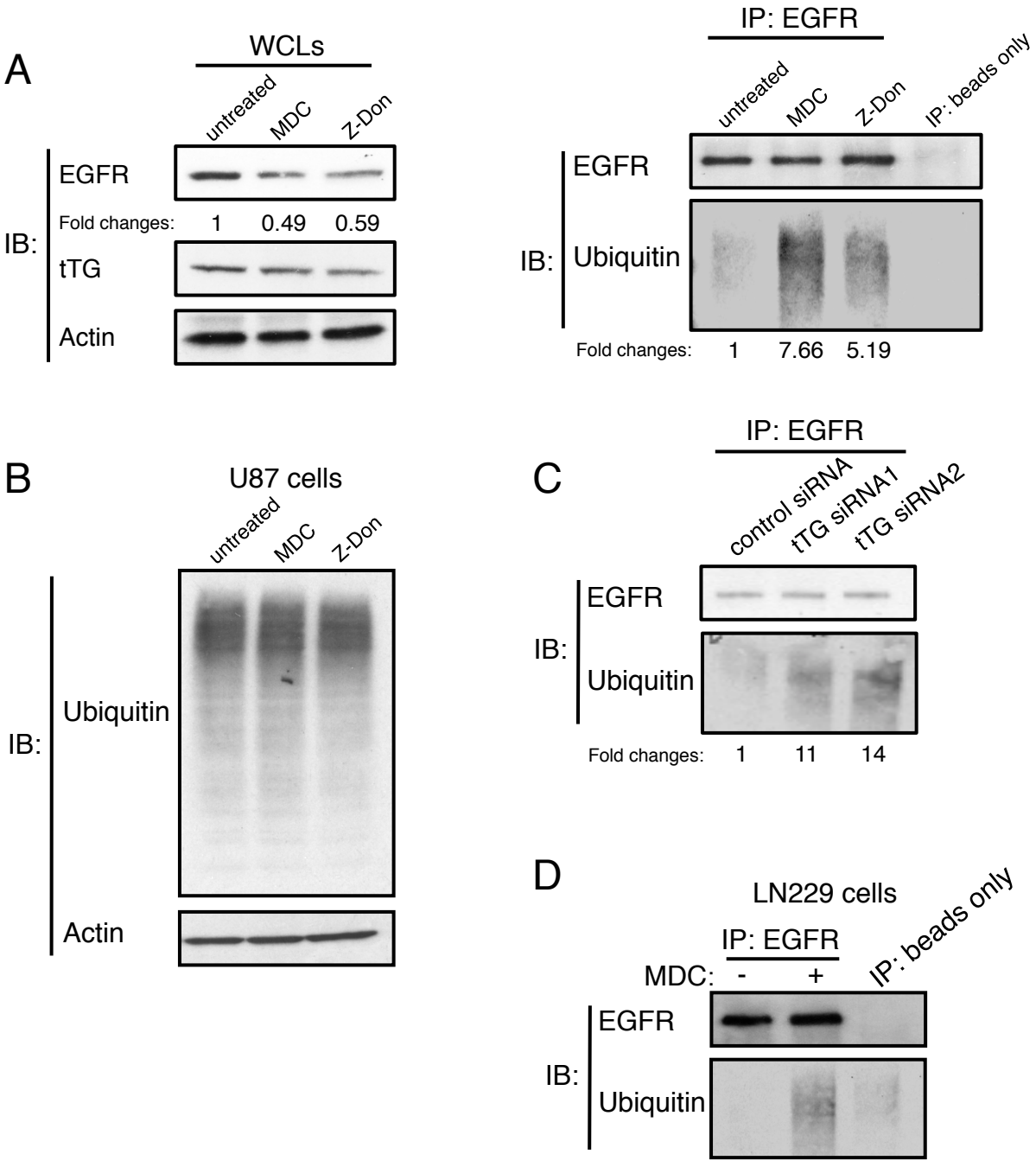
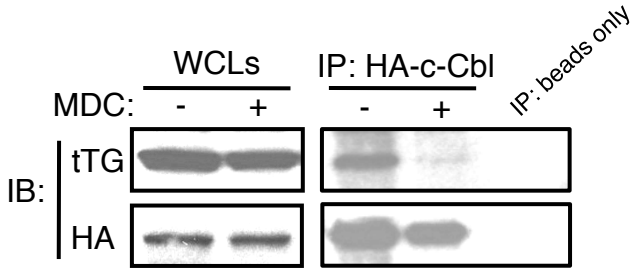

A
WCLs
untreated
MDC
Z-Don
IB:
EGFR
Fold changes:
1
0.49
0.59
tTG
Actin
IP: EGFR
untreated
MDC
Z-Don
IP: beads only
EGFR
IB:
Ubiquitin
Fold changes:
1
7.66
5.19
B
U87 cells
untreated
MDC
Z-Don
IB:
Ubiquitin
Actin
C
IP: EGFR
control siRNA
tTG siRNA1
tTG siRNA2
EGFR
IB:
Ubiquitin
Fold changes:
1
11
14
D
LN229 cells
IP: EGFR
IP: beads only
MDC:
-
+
EGFR
IB:
Ubiquitin

Figure 2.5 continued

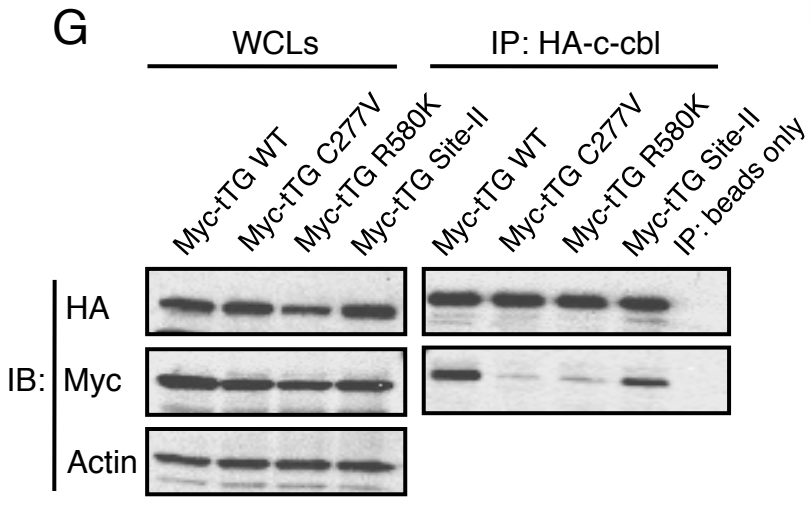
E



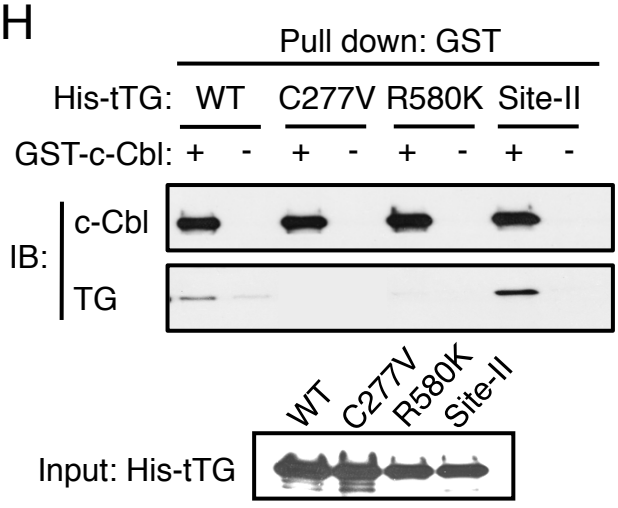
F

tTG constructs	Mutations	Nucleotide binding activity	Transamidation activity
WT	---	+++	++
C277V	Catalytic domain	+	-
R580K	Nucleotide binding domain	-	+++
Site-II (D306N, N310A)	Ca^{2+} binding domain	+++	-

G



H



ubiquitylation of the immunoprecipitated EGFR was enhanced, compared to an equivalent amount of EGFR immunoprecipitated from untreated cells. It is worth noting that the overall levels of ubiquitylated proteins in U87 cells were largely unaffected by MDC or Z-Don treatment (Figure 2.5B), suggesting that the changes in the ubiquitylation status of the EGFR caused by tTG represented a specific regulatory event. Similar increases in EGFR ubiquitylation were observed when tTG expression was knocked-down by siRNAs in U87 cells (Figure 2.5C), or when LN229 cells were exposed to MDC (Figure 2.5D).

In an effort to understand the molecular mechanism by which tTG inhibits EGFR ubiquitylation, we examined whether tTG might be capable of associating with c-Cbl in cells. Figure 2.5E shows that endogenous tTG can be co-immunoprecipitated with HA-tagged c-Cbl from U87 cells. This interaction was greatly reduced when the cells were treated with MDC. Based on these findings, our initial assumption was that the acyl transferase or transamidation activity of tTG was in some way involved in its ablity to associate with and/or negatively regulate the function of c-Cbl. Such an idea was consistent with several findings that suggested the enzymatic transamidation activity of tTG was necessary for mediating many of its effects in cells (*16, 22, 33*).

We therefore set out to further examine this possibility by using various tTG mutants that we have developed in the laboratory (Figure 2.5F). The results in Figure 2.5G (panels on the right) show that a Myc-tagged wild-type tTG construct was capable of associating with HA-tagged c-Cbl, similar to what we had observed with endogenous tTG, whereas, a transamidation-defective mutant, tTG C277V, in which the active site cysteine residue had been changed to a valine (*6*), was incapable of being co-immunoprecipitated with c-Cbl. This seemed to support the suggestion that the transamidation activity of tTG was involved in the interaction. However,

surprisingly, we found that another tTG mutant, tTG D306N, N310A (referred to as the "Site-II mutant"), that was incapable of catalyzing transamidation because of two substitutions at one of the major Ca^{2+}-binding sites essential for catalysis (*33*), was able to associate with c-Cbl (Figure 2.5G, panels on the right). Moreover, a GTP-binding-defective tTG mutant (tTG R580K), that exhibits greatly enhanced transamidation activity because its enzymatic activity is not subject to the same negative regulation that accompanies the binding of GTP to wild-type tTG (*34*), was incapable of associating with c-Cbl. Collectively, these results ruled out the possibility that the transamidation activity of tTG was required for its ability to associate with and inhibit the E3 ubiquitin ligase activity of c-Cbl.

We then asked whether the ability of tTG to associate with c-Cbl in cells was the outcome of a direct binding interaction. Purified recombinant, His-tagged wild-type tTG, as well as different tTG mutants, were incubated with purified recombinant GST-c-Cbl, and then the proteins were examined for complex formation by precipitating the GST-c-Cbl with glutathione-coated agarose beads. Figure 2.5H (first lane) shows that His-tagged wild-type tTG was co-precipitated with GST-c-Cbl, indicating that these proteins undergo a direct binding interaction. Likewise, the recombinant His-tagged tTG Site-II mutant was able to bind GST-c-Cbl, whereas His-tagged tTG R580K and His-tagged tTG C277V were ineffective (Figure 2.5H), consistent with our co-immunoprecipitation data in cells.

The GTP-bound 'closed' form of tTG is responsible for inhibiting c-Cbl function

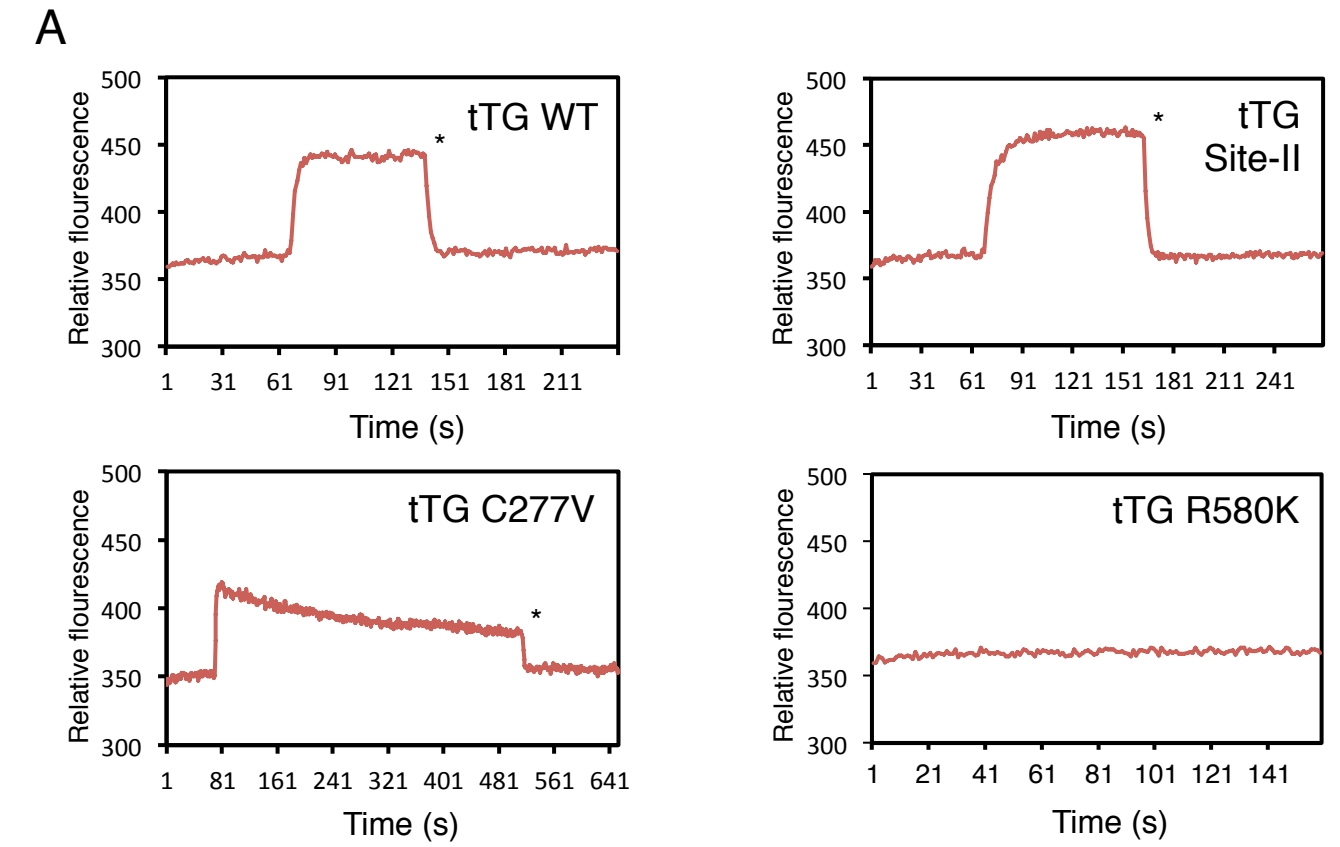
We next examined whether the GTP-binding activity of tTG is involved in its ability to associate with c-Cbl. The GTP-binding capabilities of the different tTG mutants, relative to the wild-type protein, were assayed by taking advantage of the ability of the fluorescent GTP-

analog, BODIPY-GTP, to rapidly bind tTG and undergo a significant change in its fluorescence emission (*34*). Figure 2.6A shows that, like the case for wild-type tTG (top left panel), BODIPY-GTP was capable of rapidly associating with the tTG Site-II mutant (top right panel), with the binding being sustained until the addition of unlabeled GTPγS, which caused a rapid reversal of the interaction with the labeled GTP-analog. BODIPY-GTP also showed a rapid association with the tTG C277V mutant (Figure 2.6A, bottom left panel); however, this was followed by a steady dissociation of the labeled GTP-analog, indicative of a much weaker interaction compared to either wild-type tTG or the tTG Site-II mutant. The tTG R580K mutant showed essentially no ability to bind BODIPY-GTP (Figure 2.6A, bottom right panel), consistent with our earlier studies that showed this mutant to be GTP-binding-defective (*34*).

X-ray crystallographic studies have shown that tTG can exist in two distinct conformational states. When bound to guanine nucleotide (i.e. GTP-bound in cells), tTG adopts what is referred to as a 'closed' conformational state where the β1 and β2 barrel domains fold over the central core of the protein that contains the transamidation catalytic site (*35*) (Figure 2.6B, left side). However, in the absence of bound GTP, the protein assumes a more extended or 'open' conformation in which the catalytic site is fully accessible (Figure 2.6B, right side). This was first shown when the x-ray structure was solved for tTG bound to a peptide that mimics the inflammatory gluten peptide substrate that binds to the transamidation active site (*36*). Thus, when the structural data is taken together with the results of the co-immunoprecipitation and GST pull-down experiments presented in Figures 2.5G and 2.5H, as well as the GTP-binding data in Figure 2.6A, it would appear that it is the GTP-bound, closed state of tTG that is best suited for associating with and functionally inactivating c-Cbl.

Figure 2.6. tTG adopts distinct conformations depending on whether it is bound to GTP.

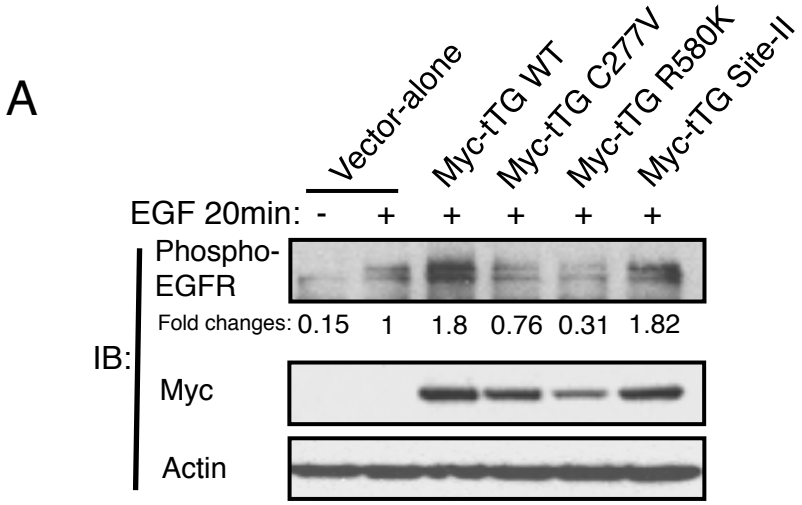
(A) Purified recombinant forms of wild-type tTG (tTG WT), tTG C227V, tTG R580K, and the tTG Site-II mutant were incubated with BODIPY-GTP and the resulting changes in fluorescence caused by the binding of BODIPY-GTP to the proteins were determined. * indicates the point in the assay when an excess of unlabeled GTPγS was added to the tTG proteins to compete off BODIPY-GTP.
(B) X-ray crystal structures and schematic representations of the closed and open conformations of tTG. tTG adopts a closed conformation when it is bound to GDP (PDB: 1KV3) (left side), or an open conformation when it is in a nucleotide free state (PDB: 2Q3Z). The domains highlighted in the tTG diagrams include the N-terminal β sandwich (N), the catalytic core (core), and the two C-terminal β-barrels (β1 and β2).
(C) Purified recombinant forms of wild-type tTG (tTG WT), tTG C277V, tTG R580K, tTG Site-II (3.0 μg of each protein) were incubated without or with trypsin for 2 hours before being resolved by SDS-PAGE and then stained with Coomassie blue to visualize the proteins.
(D) Purified recombinant wild-type tTG (3.0 μg) pre-treated without or with increasing amounts of MDC was incubated with trypsin for 2 hours before being resolved by SDS-PAGE and then stained with Coomassie blue to visualize the proteins.

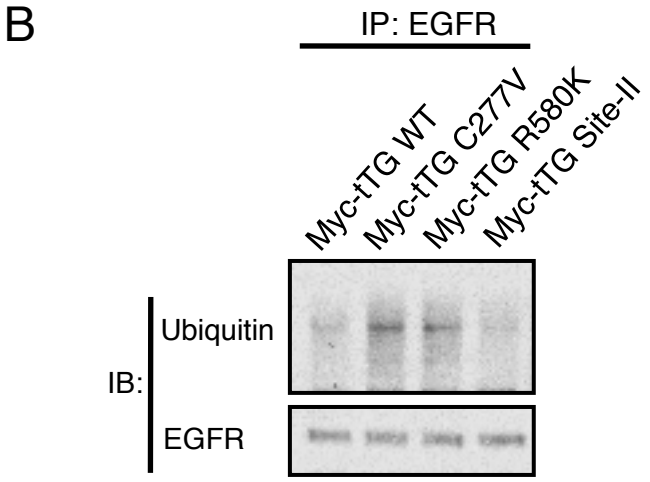

A
tTG WT
tTG Site-II
tTG C277V
tTG R580K
Relative flourescence
Time (s)
B tTG WT (closed conformation)
tTG WT (open conformation)
N
Core
β1
β2
C
tTG WT
tTG C277V
tTG R580K
tTG Site-II
Trypsin:
tTG
D
MDC(mM):
tTG WT:
Trypsin:
tTG

Further support for this idea came from studies where we examined the protease-sensitivity of wild-type tTG and the different tTG mutants. Figure 2.6C shows that wild-type tTG, and the tTG Site-II mutant, exhibited similar capabilities for resisting trypsin proteolysis, whereas, the tTG C277V mutant behaved in a similar manner to the GTP-binding-defective tTG R580K mutant and was highly sensitive to protease treatment. This indicates that the tTG Site-II mutant adopts an overall conformation similar to that of the GTP-bound, wild-type protein, as indicated by its ability to resist trypsin proteolysis, whereas the tTG C277V mutant assumes a conformation more like that of the GTP-binding-defective tTG R580K mutant. Interestingly, we found that MDC, in a dose-dependent manner, enhanced the protease sensitivity of wild-type tTG, thus causing it to behave more like the tTG R580K and tTG C277V mutants (Figure 2.6D). These findings might then explain why MDC treatment inhibits the ability of tTG to interact with c-Cbl, as read-out in co-immunoprecipitation experiments (Figure 2.5E). Specifically, rather than weakening the interaction between tTG and c-Cbl by blocking access to the transamidation active site of tTG, the binding of the inhibitor helps tTG to assume a more open conformation that has very weak affinity for c-Cbl.

The relative effectiveness of these different mutants to associate with c-Cbl could be correlated with their effects on EGFR activation (auto-phosphorylation) and receptor ubiquitylation. Figure 2.7A shows that when the different mutants were transiently expressed in U87 cells as Myc-tagged proteins, both wild-type tTG and the Myc-tTG Site-II mutant were able to increase the levels of activated EGFR upon EGF treatment, whereas the Myc-tTG C277V mutant and the GTP-binding-defective Myc-tTG R580K mutant were completely ineffective. Although we were not able to make reliable comparisons of the effects of the different mutants on EGFR ubiquitylation in U87 cells, we were able to compare their actions in the human breast

Figure 2.7. Ectopic expression of tTG forms that are in the "closed" conformation enhances EGFR signaling.
(A) Cell extracts from U87 cells ectopically expressing the vector-alone, wild-type tTG (Myc-tTG WT), or one of the different mutant forms of tTG stimulated without or with EGF (100 ng/ml) for 20 min, were immunoblotted (IB) using phospho-EGFR, Myc, and actin antibodies.
(B) Immunoprecipitations with an EGFR antibody (IP: EGFR) were performed on the cell extracts from SKBR3 cells transfected with wild-type (Myc-tTG WT) or one of the different mutant forms of tTG indicated. The resulting immuno-complexes were then immunoblotted (IB) with ubiquitin and EGFR antibodies.


A
Vector-alone
Myc-tTG WT
Myc-tTG C277V
Myc-tTG R580K
Myc-tTG Site-II
EGF 20min: - + + + + +
IB:
Phospho-EGFR
Fold changes: 0.15 1 1.8 0.76 0.31 1.82
Myc
Actin


B
IP: EGFR
Myc-tTG WT
Myc-tTG C277V
Myc-tTG R580K
Myc-tTG Site-II
IB:
Ubiquitin
EGFR

cancer cell line, SKBR3, which lacks endogenous tTG expression. The results of these experiments suggested that only wild-type tTG and the tTG Site-II mutant were able to reduce EGFR ubiquitylation (Figure 2.7B), which was fully consistent with their abilities to associate with c-Cbl.

Discussion

In this study, we set out to gain new insights into the molecular mechanisms contributing to the development of malignant gliomas. These efforts led us to tTG, a GTP-binding protein and Ca^{2+}-dependent acyl transferase, whose expression is up-regulated in several types of cancer (*6, 14, 16*). We show that this is also true for malignant gliomas. While the levels of tTG were consistently low in normal brain, as well as in most grade I and II tumors, tTG expression was noticeably up-regulated in the more aggressive, high grade (i.e. grades III and IV) tumors. In fact, we found that it was over-expressed in ~70% of these cases, making the up-regulation of tTG expression one of the most frequently occurring events in human brain tumors. Moreover, there appears to be a correlation between the increased expression of tTG in brain cancer and a poor survival prognosis for patients.

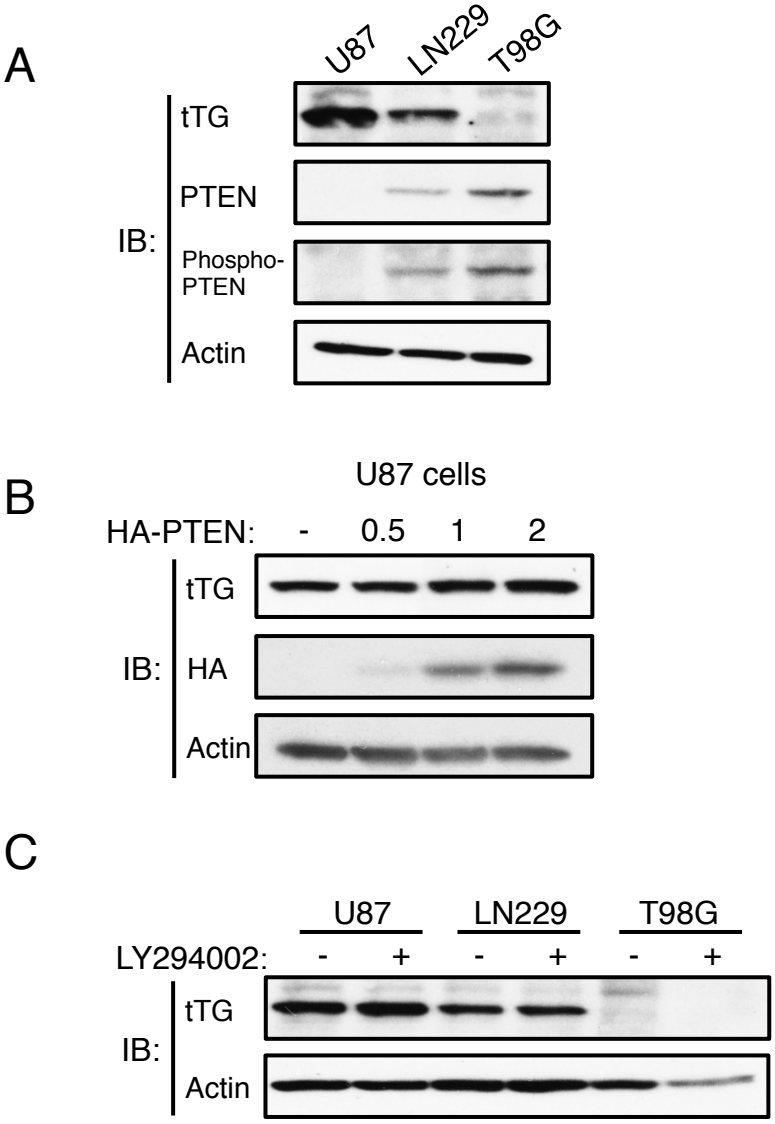
A number of studies have implicated tTG in cancer cell growth, chemoresistance, invasiveness, and metastasis (*16, 17*), and to be a downstream signaling partner of the EGFR (*6, 18, 19*). Its involvement in EGFR-signaling was particularly intriguing to us, since EGFR over-expression and activating EGFR mutations are recurring themes in human cancer and are especially important for the aggressive phenotypes exhibited by high grade primary brain tumors (*29, 37, 38*). In fact, over-expression of the EGFR has been reported in a large majority of the high grade (grade IV) tumors or glioblastomas, while it is rarely seen in the lower grade I and II

brain tumors. Consequently, EGFR status is considered to be a prognostic indicator for patients with brain tumors (*10, 21, 39*).

It has been well established that the amplification of the gene encoding the EGFR represents one common mechanism through which EGFR expression levels are increased in glioblastomas (*38*). However, 10-30% of brain cancers that show high EGFR expression contain a normal receptor gene copy number. Therefore, additional mechanisms must account for the relatively high EGFR protein levels and enhanced EGFR-signaling observed in some brain cancers (*29*). In light of these findings, we began to consider the idea that a previously unappreciated functional interplay might exist among tTG, the EGFR, and brain tumor progression. This in fact turned out to be the case. We now describe how tTG helps maintain EGFR protein levels in glioblastomas, enhancing the extent and lifetime of EGFR activation and down-stream signaling activities, through a mechanism that does not involve gene amplification but, rather, is due to the ability of tTG to interfere with receptor ubiquitylation and degradation that is normally catalyzed by the EGFR-adaptor protein and negative regulator, c-Cbl.

Work by Vivanco et al. showed that PTEN loss can result in a diminished c-Cbl-dependent degradation of EGFRs (*40*). Because previous studies from our laboratory showed that the PI3K/Akt pathway is necessary for the up-regulation of tTG expression in breast cancer cells upon growth factor treatment (*6*), we asked whether the inactivation of PTEN could regulate tTG levels and that perhaps changes in tTG expression could explain the effects of PTEN on EGFR degradation. Consistent with previous reports, U87 cells contain a truncated version of PTEN, LN229 cells express wild-type PTEN, and T98G cells contain PTEN harboring a point mutation (*41*). However, although an inverse correlation appears to exist between the expression levels of tTG and PTEN in the different glioblastoma cells that we examined (Figure

Figure 2.8. tTG expression is not regulated by PTEN.
(A) Cell extracts from U87, LN229, and T98G cells were immunoblotted (IB) with tTG, PTEN, phospho-PTEN, and actin antibodies.
(B) Cell extracts from U87 cells transiently expressing increasing amounts of HA-tagged PTEN for two days were immunoblotted with tTG, HA, and actin antibodies.
(C) Cell extracts from serum-starved U87, LN229 and T98G cells treated without or with LY294002 for 3 days were immunoblotted with tTG and actin antibodies.

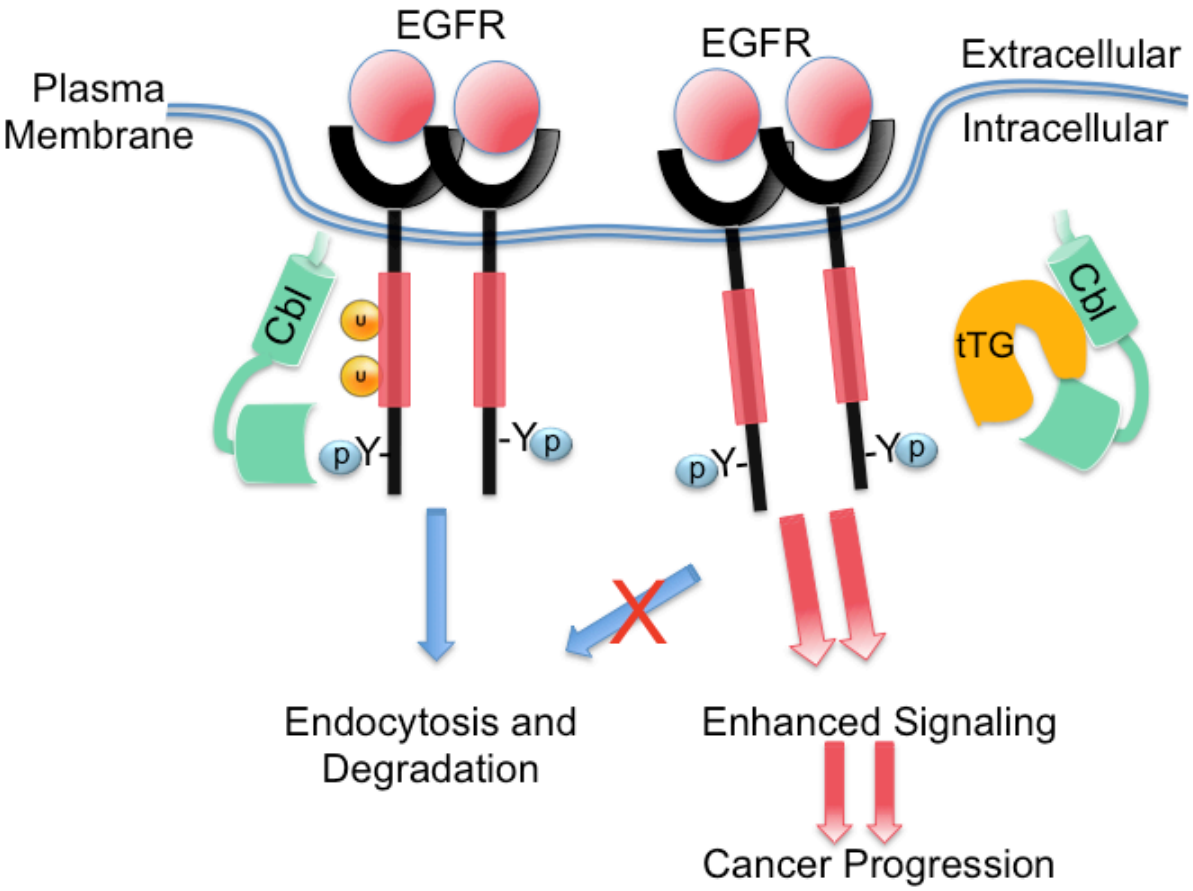

A
U87
LN229
T98G
IB:
tTG
PTEN
Phospho-
PTEN
Actin
B
U87 cells
HA-PTEN:
-
0.5
1
2
IB:
tTG
HA
Actin
C
U87
LN229
T98G
LY294002:
-
+
-
+
-
+
IB:
tTG
Actin

2.8A), we did not observe any significant change in the levels of tTG when we ectopically expressed increasing amounts of PTEN in U87 cells (Figure 2.8B) or mimicked PTEN function by inhibiting downstream signaling events with a PI3K inhibitor LY294002 (Figure 2.8C), suggesting that up-regulation of tTG in these brain cancer cells is not due to inactivation of PTEN.

tTG is best known for its enzymatic, Ca^{2+}-dependent acyl transferase activity that catalyzes the crosslinking of proteins through the formation of covalent linkages between glutamine residues on 'acceptor' proteins and lysine residues on 'donor' proteins (*17*). However, interestingly, tTG is also capable of binding and hydrolyzing GTP similar to other classical G-proteins (*17*). These two activities are reciprocally regulated, such that GTP-bound tTG exhibits little detectable transamidation activity, whereas millimolar levels of Ca^{2+} weaken GTP binding to tTG and thereby stimulate its enzymatic activity. This would imply that intracellular tTG exists in its GTP-bound state, given the high cellular concentrations of GTP and typically low concentrations of Ca^{2+}, whereas upon its secretion, tTG may then be capable of catalyzing protein crosslinking events. In fact, as schematized in Figure 2.9 and discussed further below, our data suggest it is the GTP-bound, closed state of tTG that is responsible for preventing c-Cbl from exerting its negative regulatory effects on the EGFR.

Two key findings have provided us with a mechanistic picture of how tTG blocks the actions of c-Cbl and thereby helps maintain EGFR protein levels in glioblastoma cells. U87 and LN229 human glioblastoma cell lines are highly dependent on EGFR-signaling for their transformed phenotypes. Interestingly, these cell lines also express inordinately high levels of tTG. Moreover, when tTG expression was knocked-down in these cells by siRNA,

Figure 2.9. Schematic representation depicting the effects of tTG on EGFR signaling. In normal conditions, c-Cbl is recruited to the activated EGFR and mediates its ubiquitylation. The ubiquitylated EGFR is then endocytosed and targeted for degradation in the lysosome. In brain tumor cells where tTG is abundantly expressed, tTG forms a complex with c-Cbl and prevents it from ubiquitylating the EGFR. This disrupts the normal down-regulation of the EGFR, extending its signaling lifetime and enhancing cellular transformation.


EGFR
EGFR
Extracellular
Plasma
Membrane
Intracellular
Cbl
u
u
pY-
-Yp
pY-
-Yp
tTG
Cbl
Endocytosis and
Degradation
Enhanced Signaling
Cancer Progression

corresponding reductions were observed in the expression and activation of the EGFR, as well as in the transforming capabilities of these cancer cell lines.

Perhaps even more striking were the results from experiments that took advantage of the unique qualities of the T98G glioblastoma cell line. Unlike U87 and LN229 cells, T98G cells are not dependent on the EGFR for their transforming potential. Moreover, they do not express, nor are they dependent upon, tTG for their transformed properties. However, when a Myc-tagged form of tTG was stably introduced into these cells, EGFR expression was increased between 3- and 4-fold compared to the vector-alone expressing control cells. Importantly, T98G cells over-expressing tTG were capable of forming nearly twice as many colonies in soft agar as their control counterparts, an outcome that was dependent on the enhanced EGFR activity associated with these cells. Thus, these findings demonstrate that the over-expression of tTG in at least certain brain tumor cell lines is sufficient to maintain relatively high EGFR levels and exacerbate their transformed phenotypes. Since tTG is overexpressed in several additional types of human cancer (i.e. breast, ovarian, and pancreatic cancer), it will be interesting to see whether this interplay between tTG and the EGFR holds up in these cancer types as well.

How does tTG have such profound effects on the actions of c-Cbl, and consequently, EGFR expression in glioblastomas? Given that knock-downs of tTG expression in U87 and LN229 cells, as well as the treatment of these cells with the tTG crosslinking inhibitor MDC or Z-Don, markedly increased the amounts of ubiquitylated EGFRs, we had initially assumed that the transamidation activity of tTG was responsible for its ability to inactivate the E3 ubiquitin ligase activity of c-Cbl. However, a careful analysis of the interaction of tTG with c-Cbl revealed that it did not rely upon, and indeed occurred independently of, tTG's transamidation activity, but instead appeared to be dependent on its GTP-binding capability. This is especially

interesting in light of structural studies that show the binding of GTP to tTG causes the protein to adopt a 'closed conformation' that maintains it in an enzymatically-inactive state by blocking substrate access to the catalytic domain. Under conditions of high Ca^{2+} (as might occur when tTG is extracellular), GTP dissociates, and tTG adopts an 'open conformation', in which the transamidation active site is accessible and able to catalyze the crosslinking of proteins. Thus, the only mutant form of tTG that constitutively adopts the closed state (i.e. Site-II mutant) is capable of binding to c-Cbl and blocking its ability to catalyze EGFR ubiquitylation. The ability of MDC treatment to block tTG from protecting the EGFR from c-Cbl-mediated ubiquitylation is most likely an outcome of this competitive tTG inhibitor causing the protein to adopt an open conformation which has weak affinity for c-Cbl. Similarly, the x-ray structure for Z-Don covalently linked to the active site cysteine of tTG (PDB accession # 3S3J) shows that the inhibitor-tTG complex adopts an open conformation, consistent with the ability of Z-Don to block the protective effects of tTG. Therefore, we believe that it is the GTP-bound, closed state of tTG, perhaps acting in a manner analogous to classical signaling G-proteins, that enables it to associate with c-Cbl and thereby provides a unique mode of regulation of EGFR expression (Figure 2.9).

In closing, we have identified a novel mechanism where through the actions of tTG, relatively high EGFR protein levels can be maintained, and EGFR-signaling activities enhanced and extended in human brain tumors, thus contributing to their oncogenic phenotypes. Given that a number of therapeutic strategies directed at blocking EGFR activation and function, primarily through the use of tyrosine kinase inhibitors and monoclonal antibodies, have only had limited success in the clinics, there remains an over-riding need to develop novel approaches that target the EGFR. Our finding that tTG is frequently over-expressed in high grade brain tumors,

coupled with the fact that it interferes with the proper down-regulation of the EGFR, raises the interesting possibility that developing approaches that block the ability of tTG to interact with c-Cbl may offer potentially new strategies for therapeutic intervention.

Experimental Procedures

Materials

Cell-culture reagents, EGF, Lipofectamine, Lipofectamine 2000, Protein-G agarose beads, and the control and tTG siRNAs were obtained from Invitrogen. G418, MDC, AG1478, and doxorubicin were from Calbiochem. Z-Don was from Zedira and BPA was from Pierce. Antibodies for tTG and actin were purchased from Thermo Fisher Scientific. Antibodies for EGFR, phospho-EGFR, phospho-Akt, Akt, phospho-c-Jun, c-Jun, phospho-ERK, ERK, PTEN and phospho-PTEN were from Cell Signaling. Ubiquitin and c-Cbl antibodies were from Santa Cruz, and HA and Myc antibodies were from Covance. Avidin/Biotin blocking solutions, Elite ABC reagent, and chromogen solution for immunohistochemical analysis were obtained from Vector Labs.

Cell culture, transfections, inhibitor treatments

U87 and SKBR3 cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), the LN229 and T98G cells were maintained in DMEM supplemented with 10% FBS, while NHAs were maintained in AGM (Lonza) supplemented with the BulletKit (Lonza). The expression constructs were introduced into cells using Lipofectamine, while the control and tTG siRNAs were introduced into cells using Lipofectamine 2000. T98G cells stably expressing the vector-alone or Myc-tagged tTG were selected by supplementing the growth

medium with 1 mg/ml G418. Where indicated, cells were treated with 50 μM MDC, 50 μM Z-Don, 10 μM AG1478, 1.0 μM doxorubicin, and 100 ng/ml EGF.

Tumor analysis

Tissue array slides composed of paraffin embedded sections of normal brain samples and human primary brain tumors of various grades (#GL208; U.S. Biomax) were subjected to immunohistochemical analysis. Briefly, the slides were deparaffinized by baking them at 60°C for 30 min and then rehydrating them in water containing 3% hydrogen peroxide for 30 min. After washing with phosphate buffered saline (PBS), the slides were boiled for 10 min in a 10 mM sodium citrate buffer (pH 6.0) and allowed to cool. The slides were then blocked with 2.5% horse serum and with Avidin/Biotin blocking solutions. The tissue arrays were incubated with a tTG, EGFR, or phospho-EGFR antibody, followed by a biotinylated secondary antibody and treatment with the Elite ABC reagent for 30 min. After washing with PBS, the slides were processed with chromogen solution. Lastly, the slides were dehydrated and mounted. The resulting staining obtained with each antibody was quantified using ImageJ software.

REMBRANDT (Repository of Molecular Brain Neoplasia Data) analysis

The REMBRANDT database (http://caintegrator.nci.nih.gov/rembrandt) was used to correlate tTG expression levels in brain tumors with patient survival rates. The parameters were set to include those brain tumor samples (343 gliomas or 181 glioblastomas) with a 2-fold or greater increase or a 2-fold or greater decrease in tTG expression. The results were presented as Kaplan-Meier Survival Plots.

TCGA analysis

tTG transcript levels in glioblastoma patient samples deposited in TCGA (https://tcga-data.nci.nih.gov/tcga/) were analyzed using the cBio Cancer Genomics Portal (z-score of 0.5). The percentage of patients with increased tTG levels were plotted for each glioblastoma sub-type.

Immunoblot analysis and immunoprecipitation

Cells were lysed with cell-lysis buffer (25 mM Tris, 100 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM DTT, 1 mM Na_3VO_4, 1 mM β-glycerolphosphate, 1 μg/mL leupeptin and 1 μg/mL aprotinin). The extracts were resolved by SDS-PAGE and transferred onto PVDF membranes. The membranes were incubated with primary antibodies diluted in TBST (20 mM Tris, 135 mM NaCl, and 0.02% Tween 20). Horseradish-peroxidase-conjugated secondary antibodies were used to detect the primary antibodies followed by exposure to ECL reagent. For immunoprecipitations, cell extracts (600 μg) were incubated with a particular antibody for 2 h, followed by the addition of Protein-G beads for 1 h. The beads were washed with cell-lysis buffer before being subjected to immunoblot analysis.

Soft agar colony formation assays

Cells ($3X10^3$) were suspended in growth medium supplemented without or with the indicated inhibitors and 0.3% agarose and plated on top of a layer of growth medium containing 0.6% agarose in a six-well plate. The growth medium and inhibitors were replenished on the cultures every fourth day, and after 10 days of growth the colonies that formed were counted and graphed.

Apoptotic assays

Cultures of cells were placed in serum-free medium containing various combinations of doxorubicin and MDC. After 1 day, the cells were collected and stained with DAPI for visualization by fluorescence microscopy. Cells undergoing apoptosis were identified by nuclei condensation/blebbing.

Transamidation activity assays

Cell extracts (15 μg of each) were incubated in a buffer containing 10 mM DTT, 10 mM $CaCl_2$, and 50 μM BPA for 10 min. The reactions were stopped with the addition of Laemmli sample buffer, followed by boiling, and then the proteins were resolved on a SDS-PAGE gel, transferred to PDVF membranes, and blocked overnight in BBST (100 mM boric acid, 20 mM sodium borate, 0.01% SDS, 0.01% Tween 20, and 80 mM NaCl) containing 10% bovine serum albumin. The membranes were incubated with horseradish-peroxidase-conjugated streptavidin, diluted at 1:2000 in BBST containing 5% BSA for 1 h, followed by extensive washing with BBST. The proteins that incorporated BPA were visualized on x-ray film after exposing the membranes to ECL reagent.

Cell surface protein isolation assays

Cell surface EGFR was isolated using the cell surface protein isolation kit according to the manufacturer's protocol (Pierce). Briefly, U87 cells exposed to different culturing conditions were incubated with a cell-impermeable biotinylation reagent that labels the exposed primary amines of proteins expressed on the cell surface before being lysed. The EGFR was then

immunoprecipitated from the extracts and the cell surface EGFR was detected using HRP-streptavidin.

BODIPY-GTP binding assays

Recombinant tTG (600 nM final concentration) was added into buffer containing 1 mM BODIPY-GTP, 50 mM Tris-HCl, 2 mM DTT and 1 mM EDTA. Fluorescence changes were measured using a Varian eclipse spectrofluorimeter. The excitation and emission wavelengths for BODIPY fluorescence were set at 504 nm and 520 nm, respectively.

Trypsin digestion assays

The recombinant tTG proteins (3 μg of each) were combined with 80 ng of trypsin in a buffer containing 20 mM Tris, 300 mM NaCl and 10% glycerol. The reaction was carried-out on ice for 2 h and stopped with the addition of Laemmli sample buffer, followed by boiling. The proteins were then resolved by SDS-PAGE and the gels were stained with Coomassie blue to visualize the proteins.

In vitro binding assays

Purified, recombinant His-tagged, wild-type tTG, or one of the mutant forms of tTG, was combined without or with purified, GST-tagged c-Cbl (1.0 μg of each protein) in a tube and rotated at 4°C for 2 h. Glutathione-coated agarose beads were then added to each tube for an additional 60 min, at which time the precipitated complexes were subjected to immunoblot analysis using tTG and c-Cbl antibodies.

REFERENCES

1. Furnari, F. B., Fenton, T., Bachoo, R. M., Mukasa, A., Stommel, J. M., Stegh, A., Hahn, W. C., Ligon, K. L., Louis, D. N., Brennan, C., Chin, L., DePinho, R. A., and Cavenee, W. K. (2007) Malignant astrocytic glioma: genetics, biology, and paths to treatment, *Genes & development 21*, 2683-2710.

2. Stupp, R., Mason, W. P., van den Bent, M. J., Weller, M., Fisher, B., Taphoorn, M. J., Belanger, K., Brandes, A. A., Marosi, C., Bogdahn, U., Curschmann, J., Janzer, R. C., Ludwin, S. K., Gorlia, T., Allgeier, A., Lacombe, D., Cairncross, J. G., Eisenhauer, E., and Mirimanoff, R. O. (2005) Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma, *The New England journal of medicine 352*, 987-996.

3. Jiang, D., Ying, W., Lu, Y., Wan, J., Zhai, Y., Liu, W., Zhu, Y., Qiu, Z., Qian, X., and He, F. (2003) Identification of metastasis-associated proteins by proteomic analysis and functional exploration of interleukin-18 in metastasis, *Proteomics 3*, 724-737.

4. Jiang, W. G., Ablin, R., Douglas-Jones, A., and Mansel, R. E. (2003) Expression of transglutaminases in human breast cancer and their possible clinical significance, *Oncology reports 10*, 2039-2044.

5. Kim, D. S., Choi, Y. B., Han, B. G., Park, S. Y., Jeon, Y., Kim, D. H., Ahn, E. R., Shin, J. E., Lee, B. I., Lee, H., Hong, K. M., and Kim, S. Y. (2011) Cancer cells promote survival through depletion of the von Hippel-Lindau tumor suppressor by protein crosslinking, *Oncogene 30*, 4780-4790.

6. Li, B., Antonyak, M. A., Druso, J. E., Cheng, L., Nikitin, A. Y., and Cerione, R. A. (2010) EGF potentiated oncogenesis requires a tissue transglutaminase-dependent signaling pathway leading to Src activation, *Proceedings of the National Academy of Sciences of the United States of America 107*, 1408-1413.

7. Lund-Johansen, M., Bjerkvig, R., Humphrey, P. A., Bigner, S. H., Bigner, D. D., and Laerum, O. D. (1990) Effect of epidermal growth factor on glioma cell growth, migration, and invasion in vitro, *Cancer Res 50*, 6039-6044.

8. Rousselet, E., Traver, S., Monnet, Y., Perrin, A., Mandjee, N., Hild, A., Hirsch, E. C., Zheng, T. S., and Hunot, S. (2012) Tumor Necrosis Factor-Like Weak Inducer of Apoptosis Induces Astrocyte Proliferation through the Activation of Transforming-Growth Factor-alpha/Epidermal Growth Factor Receptor Signaling Pathway, *Molecular pharmacology 82*, 948-957.

9. Moscatello, D. K., Montgomery, R. B., Sundareshan, P., McDanel, H., Wong, M. Y., and Wong, A. J. (1996) Transformational and altered signal transduction by a naturally occurring mutant EGF receptor, *Oncogene 13*, 85-96.

10. Shinojima, N., Tada, K., Shiraishi, S., Kamiryo, T., Kochi, M., Nakamura, H., Makino, K., Saya, H., Hirano, H., Kuratsu, J., Oka, K., Ishimaru, Y., and Ushio, Y. (2003) Prognostic value of epidermal growth factor receptor in patients with glioblastoma multiforme, *Cancer Res 63*, 6962-6970.

11. Umesh, S., Tandon, A., Santosh, V., Anandh, B., Sampath, S., Chandramouli, B. A., and Sastry Kolluri, V. R. (2009) Clinical and immunohistochemical prognostic factors in adult glioblastoma patients, *Clinical neuropathology 28*, 362-372.

12. Guillaudeau, A., Durand, K., Pommepuy, I., Robert, S., Chaunavel, A., Lacorre, S., DeArmas, R., Bourtoumieux, S., El Demery, M., Moreau, J. J., and Labrousse, F. (2009) Determination of EGFR status in gliomas: usefulness of immunohistochemistry and fluorescent in situ hybridization, *Applied immunohistochemistry & molecular morphology : AIMM / official publication of the Society for Applied Immunohistochemistry 17*, 220-226.

13. Libermann, T. A., Nusbaum, H. R., Razon, N., Kris, R., Lax, I., Soreq, H., Whittle, N., Waterfield, M. D., Ullrich, A., and Schlessinger, J. (1985) Amplification, enhanced expression and possible rearrangement of EGF receptor gene in primary human brain tumours of glial origin, *Nature 313*, 144-147.

14. Miyoshi, N., Ishii, H., Mimori, K., Tanaka, F., Hitora, T., Tei, M., Sekimoto, M., Doki, Y., and Mori, M. (2010) TGM2 Is a Novel Marker for Prognosis and Therapeutic Target in Colorectal Cancer, *Annals of surgical oncology 17*, 967-972.

15. Singer, C. F., Hudelist, G., Walter, I., Rueckliniger, E., Czerwenka, K., Kubista, E., and Huber, A. V. (2006) Tissue array-based expression of transglutaminase-2 in human breast and ovarian cancer, *Clin Exp Metastasis 23*, 33-39.

16. Verma, A., Wang, H., Manavathi, B., Fok, J. Y., Mann, A. P., Kumar, R., and Mehta, K. (2006) Increased expression of tissue transglutaminase in pancreatic ductal adenocarcinoma and its implications in drug resistance and metastasis, *Cancer Res 66*, 10525-10533.

17. Li, B., Cerione, R. A., and Antonyak, M. (2011) Tissue transglutaminase and its role in human cancer progression, *Advances in enzymology and related areas of molecular biology 78*, 247-293.

18. Antonyak, M. A., Li, B., Regan, A. D., Feng, Q., Dusaban, S. S., and Cerione, R. A. (2009) Tissue transglutaminase is an essential participant in the epidermal growth factor-stimulated signaling pathway leading to cancer cell migration and invasion, *The Journal of biological chemistry 284*, 17914-17925.

19. Boroughs, L. K., Antonyak, M. A., Johnson, J. L., and Cerione, R. A. (2011) A unique role for heat shock protein 70 and its binding partner tissue transglutaminase in cancer cell migration, *The Journal of biological chemistry 286*, 37094-37107.

20. Verhaak, R. G., Hoadley, K. A., Purdom, E., Wang, V., Qi, Y., Wilkerson, M. D., Miller, C. R., Ding, L., Golub, T., Mesirov, J. P., Alexe, G., Lawrence, M., O'Kelly, M., Tamayo, P., Weir, B. A., Gabriel, S., Winckler, W., Gupta, S., Jakkula, L., Feiler, H. S., Hodgson, J. G.,

James, C. D., Sarkaria, J. N., Brennan, C., Kahn, A., Spellman, P. T., Wilson, R. K., Speed, T. P., Gray, J. W., Meyerson, M., Getz, G., Perou, C. M., and Hayes, D. N. (2010) Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1, *Cancer cell 17*, 98-110.

21. Quaranta, M., Divella, R., Daniele, A., Di Tardo, S., Venneri, M. T., Lolli, I., and Troccoli, G. (2007) Epidermal growth factor receptor serum levels and prognostic value in malignant gliomas, *Tumori 93*, 275-280.

22. Antonyak, M. A., Miller, A. M., Jansen, J. M., Boehm, J. E., Balkman, C. E., Wakshlag, J. J., Page, R. L., and Cerione, R. A. (2004) Augmentation of tissue transglutaminase expression and activation by epidermal growth factor inhibit doxorubicin-induced apoptosis in human breast cancer cells, *The Journal of biological chemistry 279*, 41461-41467.

23. Qian, X., Ren, Y., Shi, Z., Long, L., Pu, P., Sheng, J., Yuan, X., and Kang, C. (2012) Sequence-Dependent Synergistic Inhibition of Human Glioma Cell Lines by Combined Temozolomide and miR-21 Inhibitor Gene Therapy, *Molecular pharmaceutics*.

24. Ren, Y., Zhou, X., Mei, M., Yuan, X. B., Han, L., Wang, G. X., Jia, Z. F., Xu, P., Pu, P. Y., and Kang, C. S. (2010) MicroRNA-21 inhibitor sensitizes human glioblastoma cells U251 (PTEN-mutant) and LN229 (PTEN-wild type) to taxol, *BMC cancer 10*, 27.

25. Weaver, K. D., Yeyeodu, S., Cusack, J. C., Jr., Baldwin, A. S., Jr., and Ewend, M. G. (2003) Potentiation of chemotherapeutic agents following antagonism of nuclear factor kappa B in human gliomas, *Journal of neuro-oncology 61*, 187-196.

26. Mizoguchi, M., Betensky, R. A., Batchelor, T. T., Bernay, D. C., Louis, D. N., and Nutt, C. L. (2006) Activation of STAT3, MAPK, and AKT in malignant astrocytic gliomas: correlation with EGFR status, tumor grade, and survival, *Journal of neuropathology and experimental neurology 65*, 1181-1188.

27. Hu, J., Jo, M., Cavenee, W. K., Furnari, F., VandenBerg, S. R., and Gonias, S. L. (2011) Crosstalk between the urokinase-type plasminogen activator receptor and EGF receptor variant III supports survival and growth of glioblastoma cells, *Proceedings of the National Academy of Sciences of the United States of America 108*, 15984-15989.

28. Antonyak, M. A., Kenyon, L. C., Godwin, A. K., James, D. C., Emlet, D. R., Okamoto, I., Tnani, M., Holgado-Madruga, M., Moscatello, D. K., and Wong, A. J. (2002) Elevated JNK activation contributes to the pathogenesis of human brain tumors, *Oncogene 21*, 5038-5046.

29. Huang, P. H., Xu, A. M., and White, F. M. (2009) Oncogenic EGFR signaling networks in glioma, *Science signaling 2*, re6.

30. Han, W., Zhang, T., Yu, H., Foulke, J. G., and Tang, C. K. (2006) Hypophosphorylation of residue Y1045 leads to defective downregulation of EGFRvIII, *Cancer biology & therapy 5*, 1361-1368.

31. Levkowitz, G., Waterman, H., Zamir, E., Kam, Z., Oved, S., Langdon, W. Y., Beguinot, L., Geiger, B., and Yarden, Y. (1998) c-Cbl/Sli-1 regulates endocytic sorting and ubiquitination of the epidermal growth factor receptor, *Genes & development 12*, 3663-3674.

32. Waterman, H., Levkowitz, G., Alroy, I., and Yarden, Y. (1999) The RING finger of c-Cbl mediates desensitization of the epidermal growth factor receptor, *The Journal of biological chemistry 274*, 22151-22154.

33. Datta, S., Antonyak, M. A., and Cerione, R. A. (2006) Importance of Ca(2+)-dependent transamidation activity in the protection afforded by tissue transglutaminase against doxorubicin-induced apoptosis, *Biochemistry 45*, 13163-13174.

34. Datta, S., Antonyak, M. A., and Cerione, R. A. (2007) GTP-binding-defective forms of tissue transglutaminase trigger cell death, *Biochemistry 46*, 14819-14829.

35. Liu, S., Cerione, R. A., and Clardy, J. (2002) Structural basis for the guanine nucleotide-binding activity of tissue transglutaminase and its regulation of transamidation activity, *Proceedings of the National Academy of Sciences of the United States of America. 99*, 2743-2747.

36. Pinkas, D. M., Strop, P., Brunger, A. T., and Khosla, C. (2007) Transglutaminase 2 undergoes a large conformational change upon activation, *PLoS Biol 5*, e327.

37. Frederick, L., Wang, X. Y., Eley, G., and James, C. D. (2000) Diversity and frequency of epidermal growth factor receptor mutations in human glioblastomas, *Cancer Res 60*, 1383-1387.

38. Layfield, L. J., Willmore, C., Tripp, S., Jones, C., and Jensen, R. L. (2006) Epidermal growth factor receptor gene amplification and protein expression in glioblastoma multiforme: prognostic significance and relationship to other prognostic factors, *Applied immunohistochemistry & molecular morphology : AIMM / official publication of the Society for Applied Immunohistochemistry 14*, 91-96.

39. Jung, Y., Joo, K. M., Seong, D. H., Choi, Y. L., Kong, D. S., Kim, Y., Kim, M. H., Jin, J., Suh, Y. L., Seol, H. J., Shin, C. S., Lee, J. I., Kim, J. H., Song, S. Y., and Nam, D. H. (2012) Identification of prognostic biomarkers for glioblastomas using protein expression profiling, *International journal of oncology 40*, 1122-1132.

40. Vivanco, I., Rohle, D., Versele, M., Iwanami, A., Kuga, D., Oldrini, B., Tanaka, K., Dang, J., Kubek, S., Palaskas, N., Hsueh, T., Evans, M., Mulholland, D., Wolle, D., Rajasekaran, S., Rajasekaran, A., Liau, L. M., Cloughesy, T. F., Dikic, I., Brennan, C., Wu, H., Mischel, P. S., Perera, T., and Mellinghoff, I. K. (2010) The phosphatase and tensin homolog regulates epidermal growth factor receptor (EGFR) inhibitor response by targeting EGFR for degradation, *Proceedings of the National Academy of Sciences of the United States of America 107*, 6459-6464.

41. Furnari, F. B., Lin, H., Huang, H. S., and Cavenee, W. K. (1997) Growth suppression of glioma cells by PTEN requires a functional phosphatase catalytic domain, *Proceedings of the National Academy of Sciences of the United States of America 94*, 12479-12484.

CHAPTER 3

Studies on the Regulation of tTG Conformation

Abstract

Tissue transglutaminase (tTG) is a dual-function acyl transferase/GTP-binding protein that has been implicated in the regulation of many physiological processes as well as in the development of various disease conditions. Previous structural and biochemical studies have revealed that tTG can exist in two conformational states, which might have important ramifications on its functions. Under normal physiological conditions, tTG is bound to GTP or GDP and adopts a closed, enzymatically-inactive configuration that has been shown to promote cell growth and survival. In contrast, in response to cellular stresses, tTG releases its bound nucleotide and assumes a more extended, open conformation. In this state, tTG is enzymatically active and has been shown to induce cell death. Given that the open and closed conformations of tTG can give rise to opposing cellular outcomes (cell survival v.s. apoptosis), we set out to better understand the molecular mechanisms that regulate the ability of tTG to adopt these two different conformations. Here we identified two pairs of intramolecular interactions that are important for maintaining tTG in its closed configuration. Specifically, two hydrogen bonds were formed between residues K677 and N681 in the most C-terminal β-barrel domain and residues W254 and D434 localized in the catalytic core domain of tTG. When these residues were mutated, it caused tTG to adopt an open conformation that triggered a strong apoptotic response in cells. These findings provide some new insights into how tTG is maintained in its closed conformation, and raise the intriguing possibility that approaches causing tTG to adopt an open conformation may be used as an effective therapy to kill cancer cells that over-express tTG.

Introduction

Tissue transglutaminase (tTG) is a member of a family of enzymes that are best known for their ability to catalyze the calcium-dependent post-translational modification of proteins by forming an irreversible amide bond between two proteins or between a protein and a polyamine (i.e. transamidation/crosslinking activity) (*1, 2*). Another function of tTG involves its ability to bind to and hydrolyze GTP, similar to other classical GTP-binding proteins (*3, 4*). Both the transamidation and GTP-binding/hydrolysis activities of tTG have been shown to participate in many physiological processes, including cellular differentiation, wound healing, and extra-cellular matrix stabilization (*5, 6*). However, aberrant tTG expression and activities have also been shown to contribute to the development of various disease conditions, including human malignancies that are characterized by chemoresistance, and neurodegenerative disorders that are characterized by cell death (*7, 8*). Therefore, understanding how tTG can impact cellular events that give rise to cell survival in some cases and cell death in others becomes an important question, especially since targeting tTG as a strategy to treat cancer and neurodegenerative disorders, such as Alzheimer's disease, Parkinson's disease and Huntington's disease, has been proposed (*9-11*).

Normally, tTG is tightly regulated at both its level of expression and activation. tTG expression in cells is typically low, but can be up-regulated by growth factor stimulation, the induction of cellular differentiation, as well as in response to various cellular insults (*12-14*). Once expressed in cells, the transamidation and the GTPase activities of tTG are subjected to further regulation. Structural studies have shown that GDP or GTP binding to tTG causes it to adopt a compact, closed conformation that prevents it from functioning as a crosslinking enzyme (*15, 16*). In this conformation, the C-terminal end of tTG folds over onto itself and blocks

substrate access to its enzymatic active site. However, under stressful conditions, decreases in cellular energy levels and increases in intracellular Ca^{2+} concentrations cause tTG to loosen its binding to nucleotide (GTP or GDP), causing the C-terminus of tTG to move away from the catalytic active site (*17, 18*). Therefore, the "open" conformation of tTG is enzymatically active and can catalyze the crosslinking reactions.

Several lines of evidence have shown that these two conformational states of tTG can have profoundly different effects on cells. For example, the closed conformation of tTG is believed to promote cellular processes that are consistent with the transformed phenotypes, including promoting cell growth and survival. This has been born out in work carried out by our laboratory and others where it was shown that the ectopic expression of wild type tTG, which primarily adopts a closed conformation, in either normal (non-transformed) or cancer cell lines protects them from apoptosis induced by serum-deprivation or chemotherapy (*19, 20*). Moreover, in the previous chapter in this thesis, I showed that tTG contributes to the oncogenic transformation of aggressive brain cancer cells by prolonging the signaling lifetime of the EGFR, and that tTG can only exert this effect when it is in the closed conformation.

On the other hand, the "open conformation" of tTG appears to have much different effects on cells. This is exemplified in experiments where expressing a GTP-binding defective form of tTG (tTG R580K), which is believed to adopt an open conformation because it is no longer susceptible to the regulation by GTP or GDP, in cells for extended periods of time induces a potent cell death response (*16*). Although this outcome was initially believed to be due to the un-regulated enzymatic activity exhibited by the tTG R580K mutant, this was subsequently proven not to be the case, as changing the active site cysteine to a valine in the tTG R580K background (thus generating an open form of tTG that is defective of crosslinking

activity) still induced cell death. Consistent with this idea, an alternatively spliced variant form of tTG called tTG-Short (tTG-S), which is often detected in the brains of Alzheimer's disease patients and exhibits greatly reduced transamidation activity, has also been shown to potently induce cell death (*21, 22*). Because this variant form of tTG lacks the C-terminal β-barrel domains, it is predicted to adopt the open conformation, thus explaining the detrimental effects it has on cells.

Here we set out to gain a deeper understanding of the mechanisms that regulate the ability of tTG to adopt the closed v.s. the open conformation. To this end, we first showed that the GTP-binding defective form of tTG, tTG R580K, indeed adopts the "open" conformation in solution using small angle x-ray scattering analysis (SAXS). We then looked for important intramolecular interactions that help keep tTG in its closed conformational state, and identified two pairs of hydrogen bonds formed between the C-terminal 15 amino acids of tTG and its catalytic core domain that were essential for maintaining tTG in the closed conformation. When we disrupted these interactions by site directed-mutagenesis, tTG was shown to constitutively adopt the open conformation and trigger an apoptotic response in cells.

Results

tTG is a multifunctional protein composed of four distinct domains, including a N-terminal β-sandwich domain, a catalytic core domain, followed by two C-terminal β-barrel domains (Figure 3.1A) (*15*). X-ray crystallographic studies revealed that tTG can exist in one of two different conformational states. When tTG is bound to nucleotide (i.e. GDP), it adopts a "closed" conformational state where the C-terminal β-barrels fold over and cover its catalytic core domain (Figure 3.1B, left panel, PDB: 1KV3) (*15*). In this state, substrate access to the

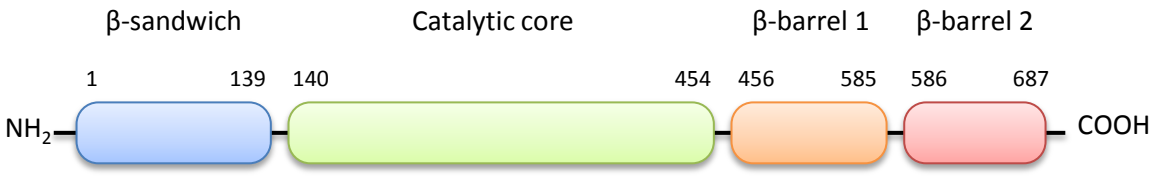
Figure 3.1. tTG adopts two different conformational states.

(A) Graphic representation of tTG. tTG is composed of four distinct domains; including a N-terminal β-sandwich (blue), a catalytic core (green), followed by β-barrel 1 (orange) and β-barrel 2 (red) at its C-terminus. The numbers indicate the amino acids of each domain.

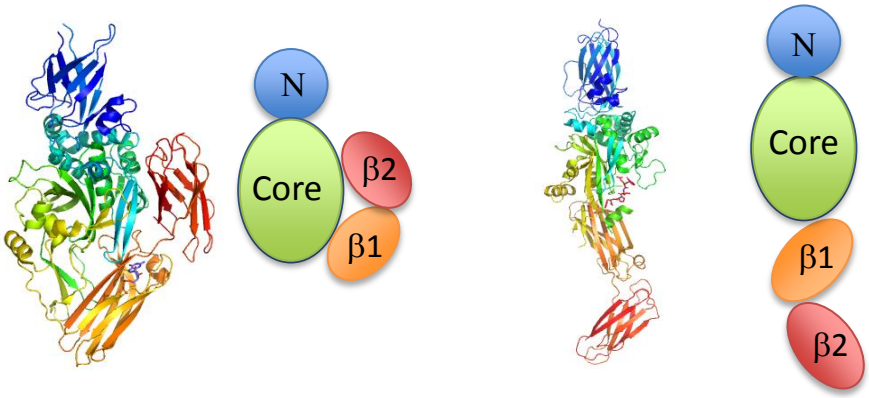
(B) The x-ray crystal structures representing the open and closed conformations of tTG. The domains in the structures are labeled as follow; the N-terminal β-sandwich (N), the catalytic core (core), and the two C-terminal β-barrels (β1 and β2). tTG adopts a closed conformation when it is bound to GDP (PDB: 1KV3) (left panel), or an open conformation when it is covalently modified with a substrate mimetic gluten peptide (PDB: 2Q3Z) (right panel). GDP and the gluten peptide are shown in sticks and colored in blue and red, respectively.

(C) Purified recombinant forms of wild type tTG (tTG WT) and tTG R580K (3.0 μg of each protein) were incubated without or with trypsin for 2 hours before being resolved by SDS-PAGE and then stained with Coomassie blue to visualize the proteins. Note that the tTG R580K mutant is more susceptible to trypsin cleavage than tTG WT.

A



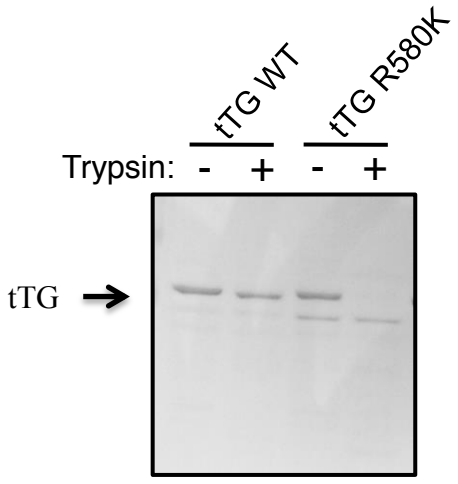
B



Closed conformation

Open conformation

C



transamidation active site of tTG is blocked, thus preventing tTG from functioning as a crosslinking enzyme. However, in another structure solved when tTG is not bound to nucleotide (GTP or GDP) and covalently modified with a substrate-mimetic gluten peptide, tTG adopts a dramatically different "open" conformation (Figure 3.1B, right panel, PDB: 2Q3Z) (*23*). In this case, the C-terminal β-barrel domains of tTG move away from the catalytic core domain and allow substrate access to the transamidation active site. Therefore, the open conformation represents the enzymatically active state of tTG.

Since the closed state of tTG promotes cell growth and survival, while the open state of tTG induces cell death, we set out to better understand the intramolecular mechanisms that influence tTG's ability to transition between these two conformations. Based on our findings shown in Figure 2.6C in the previous chapter, and again here in Figure 3.1C, recombinant wild type tTG is largely insensitive to trypsin cleavage (compare the first two lanes). This is in contrast to a form of tTG that is defective in GTP binding due to mutation of an arginine residue, essential for coordinating GTP, to a lysine residue (tTG R580K), which shows increased sensitivity to trypsin digestion (Figure 3.1C, compare the third and the fourth lane). Based on these results, we predict that wild type tTG primarily adopts a closed conformation that is insensitive to trypsin proteolysis, and tTG R580K adopts an open conformation and is more accessible for trypsin digestion.

To further verify that tTG R580K assumes an open conformation and wild type tTG adopts a closed conformation, we performed small angle x-ray scattering analysis (SAXS) on these two forms of tTG, to gain additional information about the overall size and shape of the macromolecules in solution. The *Rg* values for wild type tTG and tTG R580K were 32.7 Å and 42.5 Å respectively (Figure 3.2). Molecular weight calculations using scattering profiles from

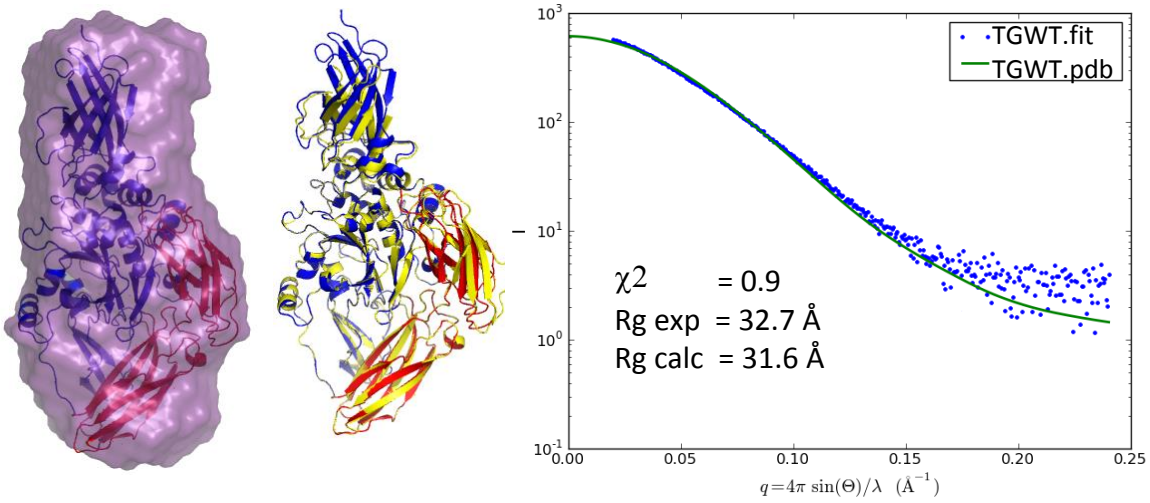
Figure 3.2. SAXS analysis on wild type tTG and the tTG R580K mutant.

(A) SAXS analysis for wild type tTG (tTG WT). A monomeric model derived from the crystal structure of GDP-bound wild type tTG (PDB: 1KV3) was fitted into the calculated SAXS envelope, with the N-terminal β-sandwich and the catalytic core domains colored in blue, and the two C-terminal β-barrel domains colored in red (left panel). A cartoon representation shows the superimposition of the fitted model (blue and red) onto the structure of wild type tTG bound to GDP (PDB:1KV3, yellow color) (middle panel). The experimental scattering profiles from SAXS are shown as blue dots, and the scattering profile for the GDP-bound wild type tTG structure (PDB: 1KV3) is shown as a green line (right panel).

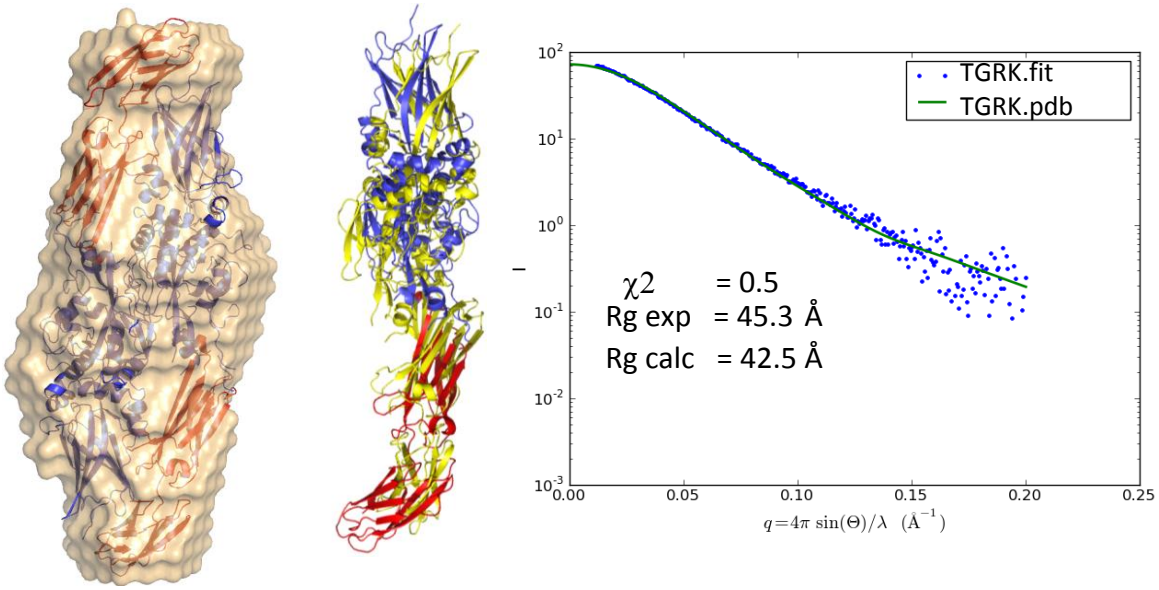
(B) SAXS analysis for the tTG R580K mutant (tTG R580K). Two monomeric models derived from a substrate-bound wild type tTG crystal structure (PDB: 2Q3Z) were fitted into the calculated SAXS envelope for tTG R580K in a head to tail fashion. The N-terminal β-sandwich and the catalytic core domains of the fitted models are colored in blue, and the two C-terminal β-barrel domains are colored in red (left panel). A cartoon representation shows the superimposition of one of the monomers in the fitted model (blue and red) onto the structure of the substrate-bound tTG (PDB: 2Q3Z, yellow color) (middle panel). The experimental scattering profiles from SAXS are shown as blue dots, and the scattering profile for the substrate-bound wild type tTG structure (PDB: 2Q3Z) is shown as a green line (right panel).

*SAXS data presented in Figure 3.2 is performed by Garima Singh.

A tTG WT



B tTG R580K



SAXS data for tTG R580K showed that it exists as a homodimer in solution with a calculated molecular weight of 153.4 kDa, while wild type tTG exists as a monomer with a calculated molecular weight of 86.1 kDa. After we applied an unbiased approach of preparing all possible models using available tTG structures and tested which of these best reflects the experimental scattering curve, we found that a monomer of a closed conformation of tTG (PDB: 1KV3) fits well for the SAXS envelope calculated for wild type tTG, with a χ^2 value of 0.9 (Figure 3.2A), and a dimer in an open conformation (PDB: 2Q3Z) fits well for the envelope calculated for the tTG R580K mutant, with a low χ^2 value of 0.5 (Figure 3.2B). These data further validated our previous finding that the guanine nucleotide-binding defective tTG R580K mutant adopts a more extended configuration, while wild type tTG assumes a more compact conformation.

It's been reported that forms of tTG that exhibit an open conformation have deleterious effects on cell viability (*16*). As shown in Figure 3.3A, ectopic expression of tTG R580K in NIH3T3 mouse fibroblasts resulted in ~20% of the cells undergoing apoptosis, as readout by nuclear condensation and blebbing. Over-expression of wild type tTG in these cells, however, had no significant effect on their apoptotic rate compared to untransfected (mock) controls. The apoptotic-promoting ability of ectopically expressed tTG R580K is accompanied by a time-dependent selection against its expression. When wild type tTG is introduced into NIH3T3 mouse fibroblast cells, its expression is detected by 12 hours after transfection, and persists in the cells for at least 2 days (Figure 3.3B, first five lanes). However, when we performed the same experiment with tTG R580K, this mutant form of tTG showed a rapid and progressive loss of expression in NIH3T3 cells. The maximal expression level of tTG R580K, which occurred 12 hours after transfection, is much lower compared to the expression level of wild type tTG at the

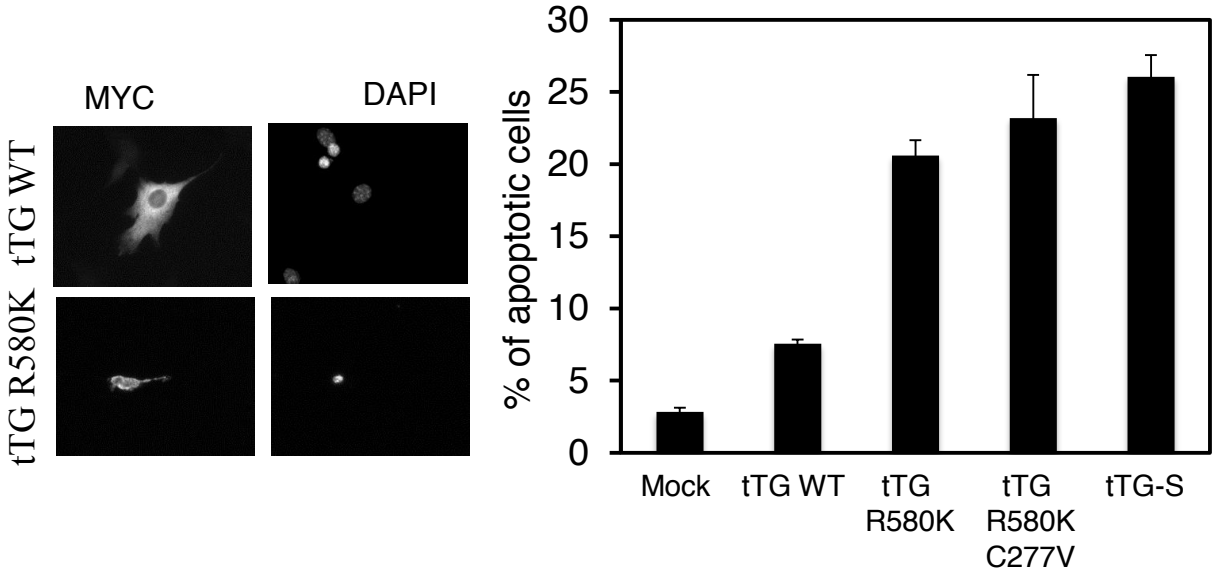
Figure 3.3. The open conformational state of tTG induces cell death.

(A) Immunofluorescence using a Myc antibody and DAPI (to stain nuclei) was performed on NIH3T3 cells that were either mock transfected or transfected with wild type tTG or one of the indicated mutant forms of tTG. Cells ectopically expressing the Myc-tagged tTG constructs were then further evaluated for apoptosis, as indicated by the presence of condensed or blebbed nuclei. Representative fluorescent pictures of cells expressing Myc-tagged wild type tTG or Myc-tagged tTG R580K are shown (left panel). Percentages of transfectants undergoing apoptosis were plotted (right panel). Data are represented as mean ± SEM.

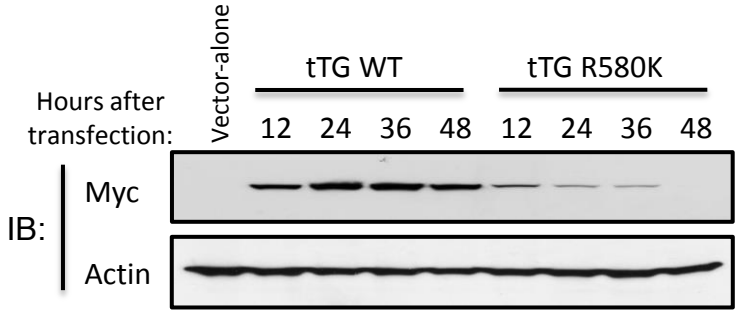
(B) Extracts from NIH3T3 cell ectopically expressing the vector-alone, wild type tTG (tTG WT), or tTG R580K were collected after the indicated hours of transfection. The extracts were then immunoblotted (IB) with Myc and actin antibodies.

(C) Cell extracts collected from 293T cells expressing different forms of Myc-tagged tTG were immunoblotted (IB) with Myc and actin antibodies (bottom two panels), as well as were assayed for their enzymatic transamidation activity, as read out by the incorporation of biotinylated pentylamine (BPA) into cellular lysate proteins (top panel).

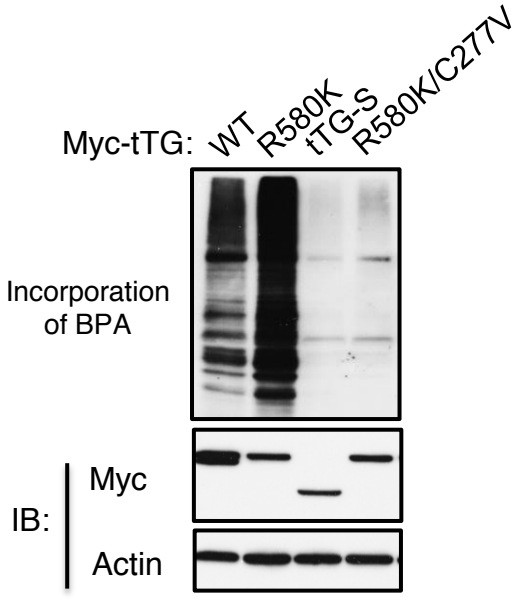
A


MYC
DAPI
tTG WT
tTG R580K
% of apoptotic cells
30
25
20
15
10
5
0
Mock
tTG WT
tTG R580K
tTG R580K C277V
tTG-S

B


Vector-alone
tTG WT
tTG R580K
Hours after transfection:
12 24 36 48 12 24 36 48
IB:
Myc
Actin

C


Myc-tTG:
WT
R580K
tTG-S
R580K/C277V
Incorporation of BPA
IB:
Myc
Actin

same time point (Figure 3.3B, compare the second and the sixth lane), and by 48 hours post transfection, no exogenous expression was detected (Figure 3.3B, last lane).

Loss of nucleotide-binding capability leads to the greatly enhanced transamidation activity exhibited by tTG R580K, compared to wild type tTG, as measured in an *in vitro* transamidation activity assay (Figure 3.3C, compare the first two lanes) (*12*). Considering that the irreversible post-translational modification of proteins catalyzed by tTG can have profound effects on their structure and function, and that the elevated transamidation activity of tTG has been associated with pathogenic protein aggregation in the development of neurodegenerative disorders, one would suspect that the apoptosis-inducing effects of tTG R580K may result from the uncontrolled crosslinking of essential proteins in cells (*24*). However, this turned out not to be the case, as a double mutant form of tTG which lacks both GTP-binding and enzymatic transamidation activities (by mutation of the essential active site cysteine to valine) (Figure 3.3C, fourth lane), referred to as tTG R580K/C277V, induced apoptosis in NIH3T3 cells to a similar extent as the tTG R580K mutant (Figure 3.3A, compare the third and fourth bars) (*20*). This indicates that the excessive transamidation activity associated with tTG R580K is not required for its cytotoxic effects. Rather, these findings suggest that it is the ability of this mutant form of tTG to adopt the open conformation that mediates its cytotoxicity.

The crystal structure of wild type tTG bound to GDP shows that the GDP molecule resides in a cleft between the catalytic core domain and the first β-barrel domain (Figure 3.1B, left panel). This hinge region undergoes a dramatic conformational change when tTG transitions from the closed conformation to the open state, as the two C-terminal β-barrels swing 120° away from the catalytic core domain to form a fully extended molecule (Figure 3.1B, right panel). Taking into account that the tTG R580K mutant is in the open conformation, it would suggest

that the nucleotide bound to tTG makes key contacts with the hinge region to help bring the C-terminal β-barrels and the catalytic core domain into close proximity, thereby stabilizing tTG in its compact conformation and inhibiting its transamidation activity.

Previous studies from our group showed that a splice variant of tTG called tTG-short (tTG-S), which lacks the C-terminal 138 amino acids, also induced a strong apoptotic response when expressed in cells (Figure 3.3A, fifth bar) (*21*). These results suggest that tTG-S likely adopts an open conformation similar to tTG R580K. Interestingly, the C-terminal region missing in tTG-S correspond to half of the first C-terminal β-barrel and all of the second β-barrel of tTG, which makes close contacts with the core domain when tTG is in its closed configuration. However, these points of contacts are absent in the open conformation due to the movement of the C-terminal β-barrels away from the catalytic core domain. This raises the possibility that the intramolecular interactions that occur between the C-terminal β-barrel 2 and the core domain of tTG may play an important role in stabilizing tTG in its closed conformation.

To test this hypothesis, we started by generating a series of tTG C-terminal truncation mutants (Figure 3.4A), with the idea being that by eliminating residues that are important for the ability of the C-terminal β-barrel to make contacts with the catalytic core, it will cause tTG to assume the open configuration. Because forms of tTG that are in the open conformation (i.e. tTG R580K and tTG-S) consistently promote apoptosis, we used this as a readout to indicate whether a given tTG mutant was in the open or closed state. Figure 3.4B showed that by truncating just the last 15 amino acids (amino acids 673-687) of tTG, we were able to generate a tTG mutant (i.e. tTG 672) that caused NIH3T3 cells to undergo apoptosis to a similar extent as the tTG R580K mutant. The C-terminal 15 amino acids of tTG correspond to the last β-sheet in the second β-barrel domain in tTG (Figure 3.4C, left panel). Close examination of the GDP-

Figure 3.4. C-terminal β-barrel 2 is involved in stabilizing tTG in its closed conformation.

(A) Schematic representation of wild type tTG and two different C-terminally truncated forms of tTG that were generated. The numbers represent the amino acids, and the truncation sites of the tTG mutants are indicated.

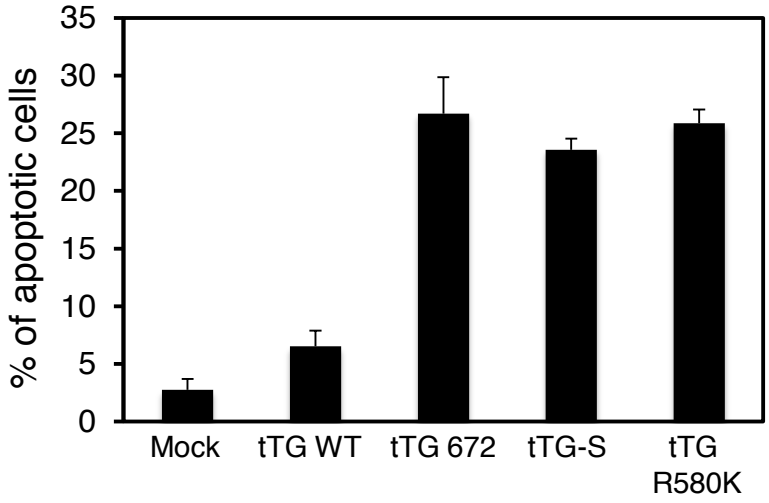
(B) Apoptotic assays were performed on NIH3T3 cells that were mock transfected or transfected with the indicated forms of tTG. The percentages of transfectants undergoing apoptosis, as read out by nuclear condensation/blebbing, were determined and then plotted. Data shown represents mean ± SEM.

(C) The C-terminal 15 amino acids of tTG (residues 673-687) are highlighted in purple in the x-ray crystal structure of tTG bound to GDP (left panel). Two pairs of hydrogen bonds formed between residues N681 and D434, and between residues K677 and W254, are shown in red with participating residues shown in sticks. Distances for each interaction are also highlighted (right panel).

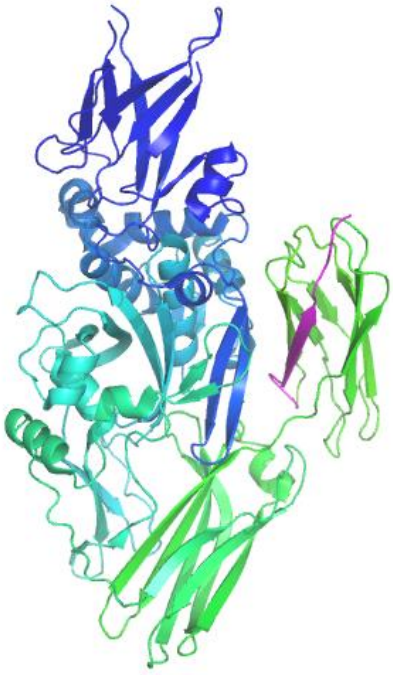
A

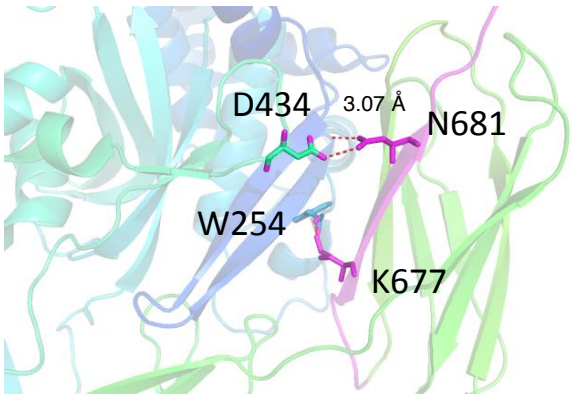

tTG WT 1 687
tTG 672 1 672
tTG-short 1 548

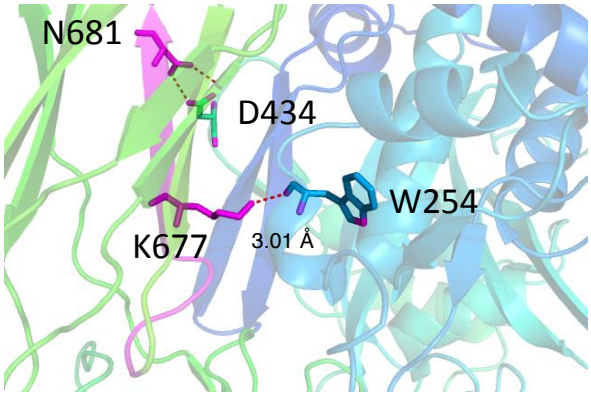
B


% of apoptotic cells
35
30
25
20
15
10
5
0
Mock
tTG WT
tTG 672
tTG-S
tTG R580K

C




D434
3.07 Å
N681
W254
K677


N681
D434
W254
K677
3.01 Å

bound tTG structure revealed that two pairs of hydrogen bonds are formed between residue K677 in the β-barrel 2 domain and residue W254 in the catalytic core domain, and between residue N681 in the β-barrel 2 domain and residue D434 in the catalytic core domain (Figure 3.4C, right panel).

To test whether the formation of these hydrogen bonds contributes to the stabilization of the closed conformation of tTG, we mutated each of the four participating residues to an alanine residue to disrupt these intramolecular interactions. We hypothesized that if these two pairs of hydrogen bonds play an important part in stabilizing tTG's closed conformation, then generating mutations that block their formation should cause tTG to assume an open conformation and induce apoptosis. Figure 3.5A shows that this was indeed the case, as ectopic expression of any one of these point mutants in NIH3T3 cells caused the cells to undergo apoptosis to a similar extent as tTG R580K, indicating that they all adopt an open conformation.

To further verify this finding, we decided to compare these mutant forms to wild type tTG and tTG R580K in trypsin proteolysis assays. Attempts to generate recombinant forms of tTG D434A and tTG K677A mutants were not successful, likely because these proteins are unstable and degrade rapidly. However, we were able to generate the recombinant forms of tTG W254A and tTG N681A, and tested their sensitivity to trypsin digestion. As shown in Figure 3.5B, both the tTG W254A and N681A mutants were almost completely digested after incubation with trypsin, confirming that they both adopt the open conformation *in vitro*.

Next, we examined whether the transamidation and GTP-binding activities of tTG were influenced by these mutations. HEK293T cells were transfected with either wild type tTG or one of the mutant forms of tTG and then were lysed. The transamidation activity associated with each of the cell extracts was then determined. Figure 3.5C shows that the W245A mutant

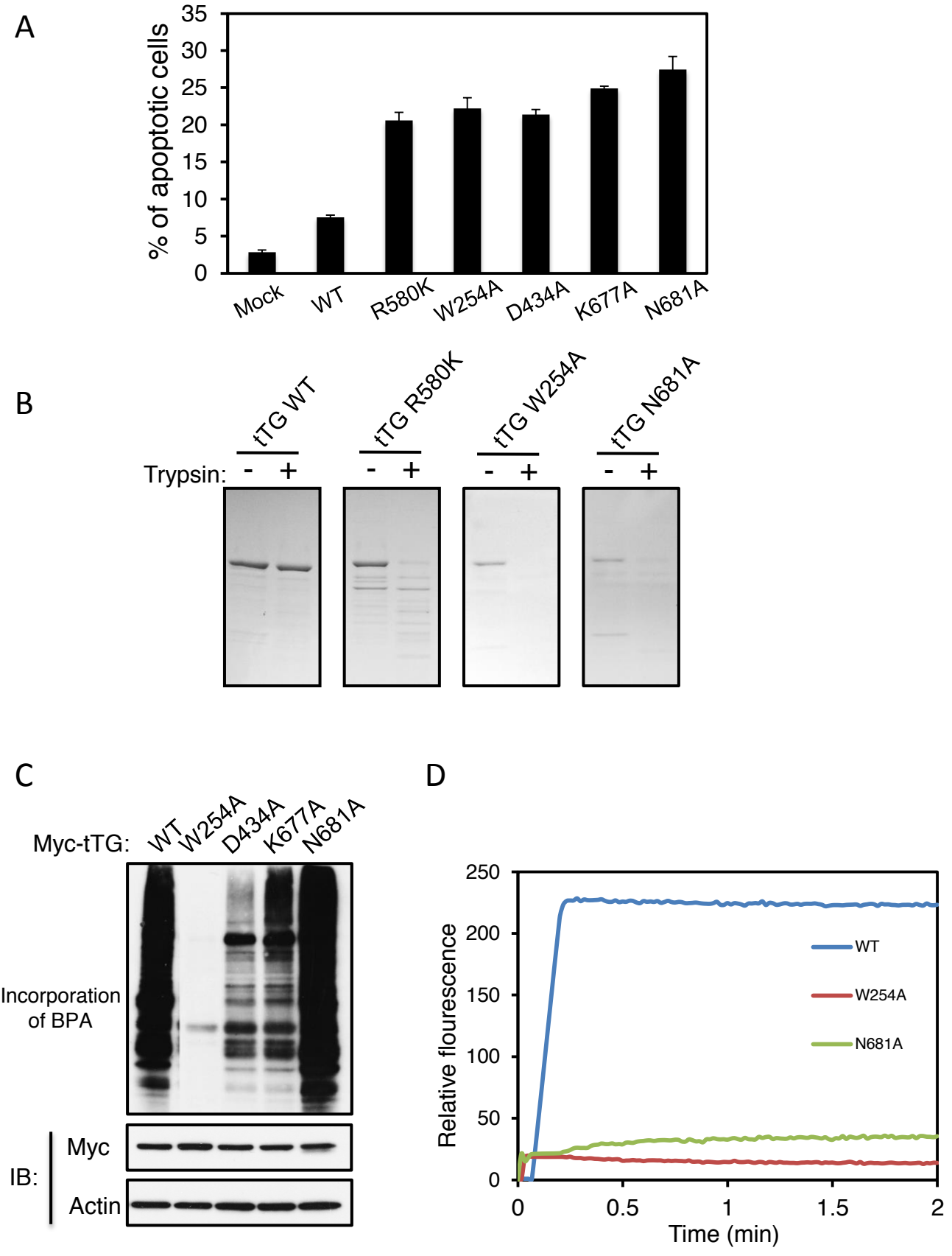
Figure 3.5. Identification of the key residues that keep tTG in a closed conformation.

(A) Apoptotic assays were performed on NIH3T3 cells that were either mock transfected or transfected with the indicated forms of tTG. The percentages of transfectants undergoing apoptosis, as read out by nuclear condensation/blebbing, were determined and then plotted. Data shown represents mean ± SEM.

(B) Purified recombinant forms of wild type tTG (tTG WT), tTG R580K, tTG W254A and tTG N681A (3.0 μg of each protein) were incubated without or with trypsin for 2 hours before being resolved by SDS-PAGE and then stained with Coomassie blue to visualize the proteins. Note that the tTG R580K, tTG W254A, and tTG N681A mutants are efficiently cleaved by trypsin, while tTG WT is not.

(C) Cell extracts collected from 293T cells ectopically expressing the different forms of Myc-tagged tTG were immunoblotted (IB) with Myc and actin antibodies (bottom two panels), as well as assayed for their enzymatic transamidation activity (top panel).

(D) Purified recombinant forms of wild type tTG (tTG WT), tTG W254A, and tTG N681A were incubated with BODIPY-GTP and the resulting changes in fluorescence caused by the binding of BODIPY-GTP to the recombinant proteins were determined and plotted. These experiments were performed at least three separate times, each yielding similar results.


A
% of apoptotic cells
35
30
25
20
15
10
5
0
Mock
WT
R580K
W254A
D434A
K677A
N681A
B
tTG WT
tTG R580K
tTG W254A
tTG N681A
Trypsin: - + - + - + - +
C
Myc-tTG: WT W254A D434A K677A N681A
Incorporation of BPA
IB: Myc
Actin
D
Relative flourescence
250
200
150
100
50
0
0
0.5
1
1.5
2
Time (min)
WT
W254A
N681A

abolished the enzymatic activity of tTG, and the D434A mutation greatly reduced it (Figure 3.5C, compare the second and third lanes to the first lane). Both W254 and D434 are localized in the catalytic core domain and are within close proximity to the catalytic active site (i.e. C277) of tTG. Thus, mutations at these two sites may perturb local structures within the catalytic domain and explain why the enzymatic activity of these mutant forms of tTG is inhibited. Mutations in the more distant C-terminal residues (i.e. tTG K677A and N681A), however, had a more modest effect on the enzymatic activity of tTG, and interestingly, the tTG N681A mutant reproducibly showed slightly elevated transamidation activity compared to wild type tTG (Figure 3.5C, compared the fourth and fifth lanes to the first lane). This suggests that the tTG N681A mutant is primarily adopting an open conformation, making the catalytic active site more accessible for substrate binding and exhibiting enhanced enzymatic activity, similar to tTG R580K (Figure 3.3C).

In addition to examining the transamidation activities of these tTG mutants, we also assayed the nucleotide-binding activities of the two available recombinant mutants (tTG W254A and tTG N681A) utilizing the fluorescent GTP analog BODIPY-GTP (*16*). Figure 3.5D shows that wild type tTG has a high affinity for BODIPY-GTP, as indicated by the significant fluorescence change upon BODIPY-GTP binding to wild type tTG, consistent with previous studies (*16*). However, both the tTG W254A mutant and the tTG N681A mutant showed little activity to bind guanine nucleotide. This suggests that, even with an intact nucleotide-binding pocket, the mutant forms of tTG that adopt the open conformational state are incapable of efficiently associating with GTP.

Discussion

Under different cellular conditions, tTG can either assume a nucleotide-bound closed conformation, or a nucleotide-free open conformation, and the two distinct conformational states of tTG are often linked to opposing cellular outcomes (cell survival v.s. cell death). In order to gain more understanding of the regulation of tTG, we set out to identify the intramolecular mechanisms that regulate the transition of tTG between these two distinct states.

X-ray structural studies predict that a nucleotide-free form of tTG is likely to assume an open conformation (*23*). We showed using trypsin proteolysis assays and SAXS analysis that a nucleotide-binding defective form of tTG, tTG R580K, indeed adopts a constitutive open conformation, while wild type tTG primarily assumes a compact, closed conformation. Previous studies showed that the ectopic expression of tTG R580K induces a strong apoptotic response in cells (*16*). Due to loss of the nucleotide-binding capability, and it adopting an open conformation, tTG R580K exhibits elevated crosslinking activity compared with wild type tTG. Considering that elevated tTG expression and activation have often been detected in several forms of neurodegenerative disorders, characterized by aggregation of pathogenic proteins, it was originally thought that the ability of tTG R580K to induce cell death resulted from the tTG-catalyzed crosslinking of essential cellular proteins in an uncontrolled manner. However, we found that the tTG R580K/C277V double mutant, which is incapable of binding GTP (and adopts an open conformation) and catalyzing transamidation activity, caused an apoptotic response similar to tTG R580K (*16*). This led us to realize that the cytotoxic phenotype exhibited by tTG R580K was not due to its uncontrolled enzymatic activity, but more likely is somehow coupled to the specific open conformation that it adopts.

Searching for other regulatory mechanisms that help to maintain tTG in the closed conformation, we turned to previous studies performed on an isoform of tTG, namely tTG-short (tTG-S). tTG-S is a naturally occurring splice variant of tTG which is identical to wild type (full length) tTG except it is lacking the C-terminal 138 amino acids. Previous studies showed that this isoform of tTG has virtually no transamidation activity and is unable to bind GTP, however, it is a strong inducer of apoptosis (*21*). This information is important for a couple of reasons. First, it provided us with another piece of evidence that the de-regulated transamidation activity is not required for the cytotoxic effects induced by tTG, as initially suspected from studies on neurodegenerative diseases, or that of the tTG R580K mutant when it is expressed in cells. Secondly, the similar cytotoxic phenotype shared by tTG-S and tTG R580K indicates that tTG-S possibly adopts a similar open configuration as tTG R580K.

Subsequently, we found that in the GDP-bound compact tTG structure, the 138 amino acids that are missing in tTG-S correspond to the C-terminal β-barrel 2 and half of the β-barrel 1, which make close contacts with the catalytic core domain of tTG. Based on this information, we proposed that the missing C-terminal end in tTG-S is involved in the stabilization of the closed conformational state of tTG. By generating a series of mutant forms of tTG, we found that two pairs of hydrogen bonds formed between residues K677 and N681 residing within the last β-sheet of tTG, with residues W254 and D434 that lie within its catalytic core domain, play an important role in maintaining tTG in its closed conformation. Mutation of any of these residues disrupted the intramolecular interactions and caused tTG to assume an open conformational state. When expressed in cells, these mutants induced apoptosis.

Overall, these findings indicate that an intricate relationship exists between the nucleotide binding status of tTG, the conformational state that it assumes, and the balance between cell

survival and death. Both nucleotide binding and the intramolecular interactions that occur between the second β-barrel of tTG (β-barrel 2) and its catalytic core domain are required to keep tTG in its compact conformation. Disrupting GDP/GTP binding by mutating the residues within the nucleotide binding pocket (i.e. tTG R580K), or disrupting the C-terminal interactions by mutating the participating residues, both lead to destabilization of the closed conformation of tTG and cause it to adopt an open state.

tTG has been shown to participate in various signaling pathways that contribute to the oncogenic transformation of cancer cells. In these cases, tTG is proposed to be in the nucleotide-bound, enzymatically inactive state, where it can function as a signaling scaffold protein and engage in different signaling pathways. Studies from my previous data chapter (Chapter 2) showed that intracellular tTG is able to extend the signaling lifetime of the epidermal growth factor receptor by interfering with its down-regulation. Other studies have shown that tTG can stimulate NFκB, Src kinase and PI3-kinase activities (*20, 25, Boroughs et al., unpublished*). All of these actions of tTG collectively lead to an array of beneficial cellular outcomes, including enhanced cell proliferation, migration, and resistance to apoptosis (*12, 26, 27*). However, how the open conformational state of tTG causes such adverse effects when expressed in cells is not fully understood. It is believed that when tTG is in the open conformation, it may selectively associate with and inactivate a cellular protein(s) that is important for maintaining cell viability, or it may activate a pro-apoptotic signaling pathway and trigger cell death. We are currently investigating these possibilities, as described further in the next chapter.

tTG is attractive to consider as a therapeutic target to treat a host of conditions/diseases including cancer and neurodegenerative disorders for several reasons. Much of this interest stems from the fact that tTG is up-regulated in disease states, but is not essential for normal

developmental processes. tTG knockout mice have no developmental defects and grow to normal size, indicating that tTG is not essential for early development nor for basic cellular functions (*28, 29*). Moreover, tTG expression is absent or occurs at relatively low levels in many normal cell lineages, whereas it is greatly up-regulated in several types of cancer and neurodegenerative disorders (*7, 18*). Therefore, eliminating abnormal tTG functions could provide a promising targeted strategy for treating these disease conditions.

Experimental Procedures

Materials

Cell-culture reagents and Lipofectamine were obtained from Invitrogen. BPA was purchased from Pierce. Actin antibody was from Thermo Fisher Scientific, and Myc antibody was from Covance.

Cell culture and transfections

HEK293T cells were maintained in DMEM medium supplemented with 10% fetal bovine serum (FBS), while NIH3T3 mouse fibroblasts were maintained in DMEM supplemented with the 10% calf serum (CS). The tTG expression constructs were introduced into cells using Lipofectamine.

Trypsin digestion assays

The recombinant tTG proteins (3 μg of each) were combined with 80 ng of trypsin in a buffer containing 20 mM Tris, 300 mM NaCl and 10% glycerol. The reaction was carried-out on ice for 2 h and stopped with the addition of Laemmli sample buffer, followed by boiling. The

proteins were then resolved by SDS-PAGE and the gels were stained with Coomassie blue to visualize the proteins.

Immunofluorescence and apoptotic assays

The cells were fixed with PBS containing 3.7% formaldehyde and then permeabilized with PBS containing 0.1% Triton X-100. After blocking in PBS containing 10% BSA, the cells were incubated with primary anti-Myc antibody for 2 h. The cells were then incubated with Oregon Green 488-conjugated secondary antibody (Molecular Probes) and DAPI (to stain nuclei) for 1 h. Following the secondary incubations, the cells were washed extensively with PBS, mounted, and visualized using the 20X objective on a Zeiss Axioskop fluorescent microscope. Cells undergoing apoptosis were identified by nuclei condensation/blebbing. The images were captured and processed using IPLAB.

Immunoblot analysis

Cells were lysed with cell-lysis buffer (25 mM Tris, 100 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM DTT, 1 mM Na_3VO_4, 1 mM β-glycerolphosphate, 1 μg/mL leupeptin and 1 μg/mL aprotinin). The extracts were resolved by SDS-PAGE and transferred onto PVDF membranes. The membranes were incubated with primary antibodies diluted in TBST (20 mM Tris, 135 mM NaCl, and 0.02% Tween 20). Horseradish-peroxidase-conjugated secondary antibodies were used to detect the primary antibodies followed by exposure to ECL reagent.

Transamidation activity assays

Cell extracts (15 μg of each) were incubated in a buffer containing 10 mM DTT, 10 mM $CaCl_2$, and 50 μM BPA for 10 min. The reactions were stopped with the addition of Laemmli sample buffer, followed by boiling, and then the proteins were resolved on a SDS-PAGE gel, transferred to PDVF membranes, and blocked overnight in BBST (100 mM boric acid, 20 mM sodium borate, 0.01% SDS, 0.01% Tween 20, and 80 mM NaCl) containing 10% bovine serum albumin. The membranes were incubated with horseradish-peroxidase-conjugated streptavidin, diluted at 1:2000 in BBST containing 5% BSA for 1 h, followed by extensive washing with BBST. The proteins that incorporated BPA were visualized on x-ray film after exposing the membranes to ECL reagent.

BODIPY-GTP binding assays

Recombinant tTG (600 nM final concentration) was added into buffer containing 1 mM BODIPY-GTP, 50 mM Tris-HCl, 2 mM DTT and 1 mM EDTA. Fluorescence changes were measured using a Varian eclipse spectrofluorimeter. The excitation and emission wavelengths for BODIPY fluorescence were set at 504 nm and 520 nm, respectively.

Small-Angle X-Ray Scattering (SAXS) Data Collection and Processing.

SAXS experiments were carried out at the Cornell High Energy Synchrotron Source (CHESS, beamline F2) at an electron energy of 10 KeV. Protein samples were prepared in 20 mM HEPES buffer (pH 7.4) containing 300 mM NaCl, 2% Glycerol and 0.5 mM TCEP at concentrations between 1 and 10 mg/ml. Ten data frames for each sample and for the corresponding buffer solution were collected. The data were corrected for background scattering, averaged, and scaled

using the program BioXtas. R_g values were estimated from the Guinier plots. Scattering data with Smax∗Rg < 1.3, computed from the Guinier plots at the low-angle regions, were used for further analysis. Kratky plots were used to assess the folded state of the proteins and overall data quality. Only data showing no sign of radiation damage or aggregation based on Guinier plots were used for further analysis. Due to concentration-dependent aggregation, only samples with concentrations between 1 mg/ml – 2.5 mg/ml were used to compose the scattering curve in the low-q range. Data were analyzed using the GNOM and CRYSOL programs. Using an indirect Fourier transform (IFT) method as implemented in the GNOM program, the intra-particle distance distribution function, p(r), Dmax, the radius of gyration of the protein, Rg, the integral properties of p(r), and the extrapolated forward scattering intensity, I (q = 0) (in a relative scale), were determined from the experimental scattering data profiles. Using the P(r) function as the target, SAXS-based shape reconstructions were carried out with the DAMMIF program. Ten independent models of each were calculated and averaged using DAMAVER. The envelopes of the reconstructed shapes were superposed onto their respective models with SUBCOMB. SASREF can minimize the discrepancy between the experimental scattering data, and the theoretical scattering curve calculated from an x-ray structural model of the individual components, thus optimizing position and orientation of the proteins. Using the SASREF program, the components of the monomer/dimer equilibrium of tissue transglutaminase (PDB: 1KV3, 2Q3Z) were treated as rigid bodies, and the modeling of domains to the SAXS data set was done through a simulated annealing protocol. SASREF simulations were computed and compared visually. CRYSOL was used to calculate the theoretical scattering curves for models where the subunits were manually positioned.

REFERENCES

1. Greenberg, C. S., Birckbichler, P. J., and Rice, R. H. (1991) Transglutaminases: multifunctional cross-linking enzymes that stabilize tissues, *Faseb J 5*, 3071-3077.

2. Folk, J. E. (1980) Transglutaminases, *Annu Rev Biochem 49*, 517-531.

3. Nakaoka, H., Perez, D. M., Baek, K. J., Das, T., Husain, A., Misono, K., Im, M. J., and Graham, R. M. (1994) Gh: a GTP-binding protein with transglutaminase activity and receptor signaling function, *Science 264*, 1593-1596.

4. Lee, M. Y., Chung, S., Bang, H. W., Baek, K. J., and Uhm, D. (1997) Modulation of large conductance Ca2+-activated K+ channel by Galphah (transglutaminase II) in the vascular smooth muscle cell, *Pflugers Arch 433*, 671-673.

5. Lorand, L., and Graham, R. M. (2003) Transglutaminases: crosslinking enzymes with pleiotropic functions, *Nature reviews. Molecular cell biology 4*, 140-156.

6. Nurminskaya, M. V., and Belkin, A. M. (2012) Cellular functions of tissue transglutaminase, *International review of cell and molecular biology 294*, 1-97.

7. Li, B., Cerione, R. A., and Antonyak, M. (2011) Tissue transglutaminase and its role in human cancer progression, *Advances in enzymology and related areas of molecular biology 78*, 247-293.

8. Jeitner, T. M., Pinto, J. T., Krasnikov, B. F., Horswill, M., and Cooper, A. J. (2009) Transglutaminases and neurodegeneration, *Journal of neurochemistry 109 Suppl 1*, 160-166.

9. Budillon, A., Carbone, C., and Di Gennaro, E. (2013) Tissue transglutaminase: a new target to reverse cancer drug resistance, *Amino acids 44*, 63-72.

10. Iannaccone, M., Stefanile, A., Vivo, G. D., Martin, A., Serretiello, E., and Gentile, V. (2012) Transglutaminase inhibition: A therapy to protect cells from death in neurodegeneration?, *World journal of biological chemistry 3*, 184-186.

11. Wilhelmus, M. M., van Dam, A. M., and Drukarch, B. (2008) Tissue transglutaminase: a novel pharmacological target in preventing toxic protein aggregation in neurodegenerative diseases, *European journal of pharmacology 585*, 464-472.

12. Antonyak, M. A., Li, B., Regan, A. D., Feng, Q., Dusaban, S. S., and Cerione, R. A. (2009) Tissue transglutaminase is an essential participant in the epidermal growth factor-stimulated signaling pathway leading to cancer cell migration and invasion, *The Journal of biological chemistry 284*, 17914-17925.

13. Antonyak, M. A., Singh, U. S., Lee, D. A., Boehm, J. E., Combs, C., Zgola, M. M., Page, R. L., and Cerione, R. A. (2001) Effects of tissue transglutaminase on retinoic acid-induced

cellular differentiation and protection against apoptosis, *The Journal of biological chemistry 276*, 33582-33587.

14. Jang, G. Y., Jeon, J. H., Cho, S. Y., Shin, D. M., Kim, C. W., Jeong, E. M., Bae, H. C., Kim, T. W., Lee, S. H., Choi, Y., Lee, D. S., Park, S. C., and Kim, I. G. (2010) Transglutaminase 2 suppresses apoptosis by modulating caspase 3 and NF-kappaB activity in hypoxic tumor cells, *Oncogene 29*, 356-367.

15. Liu, S., Cerione, R. A., and Clardy, J. (2002) Structural basis for the guanine nucleotide-binding activity of tissue transglutaminase and its regulation of transamidation activity, *Proceedings of the National Academy of Sciences of the United States of America 99*, 2743-2747.

16. Datta, S., Antonyak, M. A., and Cerione, R. A. (2007) GTP-binding-defective forms of tissue transglutaminase trigger cell death, *Biochemistry 46*, 14819-14829.

17. Casadio, R., Polverini, E., Mariani, P., Spinozzi, F., Carsughi, F., Fontana, A., Polverino de Laureto, P., Matteucci, G., and Bergamini, C. M. (1999) The structural basis for the regulation of tissue transglutaminase by calcium ions, *European journal of biochemistry / FEBS 262*, 672-679.

18. Jeitner, T. M., Muma, N. A., Battaile, K. P., and Cooper, A. J. (2009) Transglutaminase activation in neurodegenerative diseases, *Future neurology 4*, 449-467.

19. Antonyak, M. A., Miller, A. M., Jansen, J. M., Boehm, J. E., Balkman, C. E., Wakshlag, J. J., Page, R. L., and Cerione, R. A. (2004) Augmentation of tissue transglutaminase expression and activation by epidermal growth factor inhibit doxorubicin-induced apoptosis in human breast cancer cells, *The Journal of biological chemistry 279*, 41461-41467.

20. Li, B., Antonyak, M. A., Druso, J. E., Cheng, L., Nikitin, A. Y., and Cerione, R. A. (2010) EGF potentiated oncogenesis requires a tissue transglutaminase-dependent signaling pathway leading to Src activation, *Proceedings of the National Academy of Sciences of the United States of America 107*, 1408-1413.

21. Antonyak, M. A., Jansen, J. M., Miller, A. M., Ly, T. K., Endo, M., and Cerione, R. A. (2006) Two isoforms of tissue transglutaminase mediate opposing cellular fates, *Proceedings of the National Academy of Sciences of the United States of America 103*, 18609-18614.

22. Citron, B. A., Suo, Z., SantaCruz, K., Davies, P. J., Qin, F., and Festoff, B. W. (2002) Protein crosslinking, tissue transglutaminase, alternative splicing and neurodegeneration, *Neurochemistry international 40*, 69-78.

23. Pinkas, D. M., Strop, P., Brunger, A. T., and Khosla, C. (2007) Transglutaminase 2 undergoes a large conformational change upon activation, *PLoS Biol 5*, e327.

24. Grosso, H., and Mouradian, M. M. (2012) Transglutaminase 2: biology, relevance to neurodegenerative diseases and therapeutic implications, *Pharmacology & therapeutics 133*, 392-410.

25. Verma, A., and Mehta, K. (2007) Transglutaminase-mediated activation of nuclear transcription factor-kappaB in cancer cells: a new therapeutic opportunity, *Current cancer drug targets 7*, 559-565.

26. Verma, A., and Mehta, K. (2007) Tissue transglutaminase-mediated chemoresistance in cancer cells, *Drug resistance updates : reviews and commentaries in antimicrobial and anticancer chemotherapy 10*, 144-151.

27. Boroughs, L. K., Antonyak, M. A., Johnson, J. L., and Cerione, R. A. (2011) A unique role for heat shock protein 70 and its binding partner tissue transglutaminase in cancer cell migration, *The Journal of biological chemistry 286*, 37094-37107.

28. Nanda, N., Iismaa, S. E., Owens, W. A., Husain, A., Mackay, F., and Graham, R. M. (2001) Targeted inactivation of Gh/tissue transglutaminase II, *The Journal of biological chemistry 276*, 20673-20678.

29. De Laurenzi, V., and Melino, G. (2001) Gene disruption of tissue transglutaminase, *Molecular and cellular biology 21*, 148-155.

CHAPTER 4

Conclusions

Increasing amounts of evidence suggest that tTG, a dual function acyl transferase/GTPase, contributes to the progression of various types of human cancers. The expression level of tTG is up-regulated in breast, pancreatic and colon cancers, and over-expression/activation of tTG has been shown to promote cancer cell migration, proliferation and survival (*1-5*). In this thesis, I set out to expand upon our understanding of how tTG contributes to the malignant transformation of cancer cells.

In the first study presented in Chapter 2, I delineated a novel mechanism through which tTG promotes brain tumor progression. In this study, I showed that tTG expression is up-regulated in 70% of high grade brain tumors, making it one of the most commonly de-regulated genes in this type of cancer. Moreover, tTG expression was negatively correlated with patient survival. Elevated expression level of tTG was also detected in several human glioblastoma cell lines, where it was shown to play an indispensible role in their transforming capabilities. We then discovered that tTG contributes to the transformed phenotypes of these aggressive brain cancer cells by extending the signaling lifetime of the EGFR.

Aberrant EGFR activation is a hallmark of glioblastomas (*6, 7*). Gene amplification and/or activating mutations of the EGFR can account for the excessive signaling activities of the EGFR in some glioblastomas (*8*). However, these mechanisms can not explain EGFR over-expression and/or hyper-activation in all cases, indicating that additional mechanisms that give rise to enhanced EGFR expression and signaling activity must exist. We found that tTG is responsible for one such mechanism through its ability to attenuate EGFR down-regulation.

EGFR ubiquitylation mediated by the E3-ubiquitin ligase c-Cbl is an important step in the down-regulation of the activated EGFR (*9, 10*). We showed that tTG can bind to c-Cbl both *in vitro* and *in vivo*, and functionally inactivate c-Cbl's E3-ubiquitin ligase activity.

Studies from several groups, including our laboratory, have reported mechanisms where enhanced EGFR signaling is achieved by disrupting the down-regulation of the EGFR. Phosphatase and tensin homolog (PTEN) and Ephrin receptor A5 (EphrinA5) are both tumor suppressors which are frequently downregulated or deleted in primary brain tumors (*11, 12*). Studies showed that PTEN and EphA5 can stabilize the complex formation between the EGFR and c-Cbl, therefore promoting the ubiquitylation and down-regulation of the activated EGFRs. However, the detailed mechanisms by which PTEN and EphA5 regulate c-Cbl function are not known. Our group reported that the Cool-1 (cloned-out of library 1) protein, a signaling molecule downstream of the EGFR, also plays a key role in regulating EGFR degradation (*13*). EGFR-dependent phosphorylation of Cool-1 enables it to function as an activator of Cdc42, which can then form a ternary complex with Cool-1 and c-Cbl, and delay c-Cbl-catalyzed EGFR down-regulation. This complex is normally transient, however, sustained Cool-1 phosphorylation was observed when a constitutively active form of the Src kinase was introduced into cells, which led to defective EGFR turnover and aberrant growth.

In our studies, we not only identified tTG as a key regulator of EGFR signaling and brain tumor cellular transformation, but we also uncovered the mechanism by which tTG affects c-Cbl function. The association between tTG and c-Cbl occurs only when tTG adopts a specific closed conformational state, i.e. when tTG is bound to GDP or GTP. Mutant forms of tTG that adopt an open conformational state, or treatment of tTG with certain inhibitors that induce the open state,

disrupt the complex formation between tTG and c-Cbl and block the ability of tTG to enhance EGFR signaling activities.

While it has been known for some time that tTG can function as a downstream target of the EGFR and is important for the growth and survival activities of the EGFR, the findings showing that tTG can regulate EGFR signaling lifetime was quite unexpected. They also raise the question as to whether the excessive EGFR signaling activities observed in these brain cancer cells are responsible for the elevated expression and activation of tTG, which in effect would give rise to a feed-back loop. One piece of evidence that supports this idea comes from a proteomic study that was published a few years ago (*14*). In this study, the protein expression profiles were compared between U87 glioma cells stably expressing either a vector-only control or the constitutively active and highly oncogenic EGFRvIII. tTG was found to be one of only four proteins whose expression were significantly up-regulated by EGFRvIII. This would suggest that the constitutive EGFR signaling activity observed in these glioblastoma cells contributes at least in part to the up-regulation of tTG expression. Possibly by increasing tTG expression in cells and blocking the actions of c-Cbl, the EGFR develops a mechanism to maintain its signaling potential in these aggressive brain tumors.

A few other interesting questions also arise from this study. First, does tTG have a broader impact and influence the half-life of other receptor tyrosine kinases besides EGFR in brain tumors? The fact that tTG has no significant influence on the overall ubiquitylation levels in these glioblastoma cells suggests that the tTG-mediated inhibition of ubiquitylation is a targeted process. The finding that tTG exerts its impact primarily on the E3-ubiqutin ligase c-Cbl further supports this idea, as the specificity of the ubiquitylation processes is primarily determined at the level of the E3-ubiquitin ligase and substrate recognition (*15*). However, c-Cbl

has been reported to function as the E3-ubiquitin ligase that down-regulates several other receptor tyrosine kinases and non-receptor tyrosine kinases, including the PDGFR, c-Met and Fyn, all of which have been implicated in glioblastoma (*16-21*). Whether tTG is capable of modulating the signaling activities of these tyrosine kinases remains to be determined. Also, whether the ability of tTG to regulate EGFR signaling holds true in cancer types other than brain tumors (i.e. breast cancer) needs to be examined.

We showed that tTG was only able to associate with c-Cbl and interfere with its ubiquitin ligase function when it is bound to nucleotide (GTP or GDP) and adopts the specific closed conformation. This finding reminded us of the less studied GTP-binding/hydrolysis activity of tTG. tTG, identified as a high molecular weight G-protein in the early 1990s, has been shown to mediate the signaling activities of a few cell surface receptors (*22, 23*). The GTPase activity of tTG has not been extensively studied, partially due to the lack of similarity between tTG and the more traditional small GTPases or the large hetero-trimeric G-proteins. At this time, no regulatory factors (GEFs or GAPs) have been identified for tTG. However, what is in common between tTG and other GTP-binding proteins is that nucleotide association induces conformational changes within the molecule that have important ramifications for their functions. In the case of the traditional small GTPases or hetero-trimeric G-proteins, the binding of nucleotides stabilizes the protein conformation, and cycling between the GTP- and GDP-bound states causes local movement of conformationally-sensitive (switch) regions that are important for engaging or disengaging upstream receptors or downstream effectors. In the case of tTG, nucleotide-free tTG and nucleotide-bound tTG adopt dramatically different configurations, as demonstrated by the two crystal structures for the GDP-bound and the nucleotide-free forms of wild type tTG (*24, 25*). Moreover, the trypsin proteolysis studies that

we performed on wild type tTG and tTG R580K mutant further demonstrated that tTG adopts a distinct conformation when it is bound to nucleotide.

Under normal physiological conditions, intracellular tTG is most likely associated with GTP/GDP due to the high concentrations of GTP present in the cytosol, therefore it is most likely to be in the closed conformation. However, under stressful conditions like those accompanying neurodegenerative disorders, energy depletion, loss of Ca^{2+} homeostasis, and the binding of Ca^{2+} to tTG cause a release of nucleotide, thus enabling tTG to adopt the open conformation. Previous studies, as well as findings presented in Chapter 2, showed that the two conformational states of tTG are often engaged in different signaling pathways, leading to distinct cellular outcomes (cell survival v.s. cell death) (*1, 26*). In chapter 3, we went on to further examine the regulatory mechanisms that control the transition of tTG between the two different states.

In addition to nucleotide-binding regulating the opening and closing of tTG, we also identified intramolecular interactions that influence these conformational transitions. Specifically, two pairs of hydrogen bonds formed between the β-barrel 2 domain and the catalytic core domain of tTG were shown to stabilize the closed conformation of tTG. Disruption of these interactions, either by individually mutating the residues that are involved in making these hydrogen bonds, or by deleting this entire region as occurs in tTG-S, a splice variant expressed in neurodegenerative disorders, resulted in forms of tTG that adopt an open conformation and induce cell death, a phenotype similar to the GTP-binding defective tTG (tTG R580K) mutant, which we have shown by SAXS analysis to also adopt an open conformation.

How the open form of tTG promotes apoptosis is still unclear. The increased crosslinking activity of tTG due to the loss of GTP binding observed with the tTG R580K

mutant, together with the aggregation of pathogenic proteins detected in neurodegenerative disorders, suggested that the uncontrolled crosslinking activity might be responsible for the ability of tTG to induce cell death. However, we proved that this cannot explain the apoptotic effects of the GTP-binding defective tTG (tTG R580K) mutant, as the R580K/C277V double mutant (that lacks both GTP-binding and crosslinking activities) induced cell death to a similar extent as tTG R580K, indicating that other unique capabilities that tTG acquires when it adopts the open conformation contributes to its cytotoxicity. We now suspect that when tTG assumes its extended conformation, it might become capable of associating with and functionally inactivating proteins that are essential for maintaining cell viability, therefore disrupting normal cellular processes and promoting cell death.

To test this idea, we are now looking for proteins that preferentially associate with the open form of tTG (i.e. tTG R580K), using two different approaches. In one such approach, we performed pull-down experiments using recombinant forms of wild type tTG and tTG R580K, representing two different states of tTG, and screened for specific binding partners in NIH3T3 fibroblast cell lysates. We found that a protein with an apparent molecular weight of ~120 kDa preferentially associated with tTG R580K in these experiments. The protein band was excised from the gel and analyzed by mass spectrometry. The top hits from the mass spectrometry analysis are listed in Table 4.1, and some them are potentially interesting with regard to the apoptotic-inducing actions of tTG R580K. For example, the #1 hit, FERM domain containing 4A (FRMD4A), and the #10 hit, FERMRhoGEF, both contain the FERM (4.1, Ezrin, Radixin and moesin) domain, which is a protein module implicated in localizing proteins to the plasma membrane (*27, 28*). This indicates that the FERM domain may represent a high-affinity binding motif for the open form of tTG. Moreover, recent studies have reported the role of FRMD4A in

Table 4.1. The identified components of the ~120 kDa band that preferentially associated with tTG R580K from mass spectrometry.

prot_hit num	prot_acc	prot_desc	prot score	prot mass	prot cover	prot pi	emPAI	molar %
1	gi|40254210	FERM domain containing 4A [Mus musculus]	1344	115771	38.4	9.09	1.12	2.95
2	gi|6678826	minichromosome maintenance deficient 2 mitotin [Mus musculus]	1253	102668	37.2	5.52	1.26	3.31
3	gi|26986595	ring finger protein 40 [Mus musculus]	1140	114835	34.8	6.11	1.19	3.13
4	gi|33859650	membrane bound C2 domain containing protein [Mus musculus]	901	121707	34.2	5.63	1.05	2.76
5	gi|16716569	protease, serine, 1 [Mus musculus]	886	26802	16.7	4.75	1.02	2.68
6	gi|33859829	ring finger protein 20 [Mus musculus]	855	114135	22.7	5.74	0.62	1.63
7	gi|111607496	Janus kinase 1 [Mus musculus]	733	135149	24.5	7.48	0.73	1.92
8	gi|33942089	ataxin 2-like [Mus musculus]	686	110865	21.2	8.94	0.79	2.08
9	gi|85662406	cytoplasmic linker 2 isoform b [Mus musculus]	662	112300	28	6.13	0.68	1.79
10	gi|110349752	FERMRhoGEF (Arhgef) and pleckstrin domain protein 1 [Mus musculus]	625	119599	30.1	7.88	0.67	1.76
11	gi|124358959	TAO kinase 1 [Mus musculus]	609	116434	22.2	7.14	0.65	1.71
12	gi|145966692	keratin complex 1, acidic, gene 1 [Mus musculus]	535	48911	23.6	4.87	1.19	3.13
13	gi|149262435	PREDICTED: keratin complex 1, acidic, gene 3 [Mus musculus]	506	55405	19.5	5.21	0.78	2.05
14	gi|13386238	keratin 34 [Mus musculus]	489	46185	22.4	4.76	1.29	3.39
15	gi|149254026	PREDICTED: SEC31-like 1 [Mus musculus]	482	145123	15.6	8.09	0.28	0.74
16	gi|112983636	keratin complex 1, acidic, gene 10 [Mus musculus]	477	57178	12.5	5	0.4	1.05
17	gi|13386274	RIKEN cDNA 2310015J09 [Mus musculus]	419	47661	21.8	4.76	1.09	2.87
18	gi|126116585	keratin complex 2, basic, gene 1 [Mus musculus]	401	66079	4.9	8.39	0.34	0.89
19	gi|149261084	PREDICTED: TBP-interacting protein isoform 1 [Mus musculus]	373	160752	16.4	6.86	0.33	0.87
20	gi|33563246	calpastatin [Mus musculus]	369	81620	24.5	5.24	0.37	0.97
21	gi|7305031	erythrocyte protein band 4.1-like 3 [Mus musculus]	355	103731	22.7	5.2	0.45	1.18
22	gi|8393684	keratin 35 [Mus musculus]	337	48984	20.7	4.76	0.92	2.42
23	gi|9055218	formin binding protein 3 [Mus musculus]	336	108698	13.6	7.39	0.39	1.03
24	gi|31980832	keratin complex 2, basic gene 18 [Mus musculus]	321	57377	23.9	6.19	1.06	2.79
25	gi|6679134	neuropilin 1 [Mus musculus]	315	104266	12.7	5.67	0.28	0.74
26	gi|20911031	keratin 5 [Mus musculus]	295	61957	16.7	7.59	0.51	1.34
27	gi|30794206	splicing factor 3b, subunit 2 [Mus musculus]	291	98237	21.6	5.46	0.39	1.03
28	gi|84781771	trypsin 10 [Mus musculus]	289	26888	18.7	5.5	0.42	1.10
29	gi|71043961	trypsinogen 7 [Mus musculus]	282	27089	4.9	8.22	0.26	0.68
30	gi|6678790	mannosidase 2, alpha 1 [Mus musculus]	282	132247	10.4	8.17	0.22	0.58

regulating cell polarity and promoting squamous cell carcinoma growth (*29, 30*). It is worth examining whether the open form of tTG can disrupt cell polarity or Rho GTPase signaling by binding to these FERM domain-containing proteins. A few other well-studied signaling molecules, including the #11 hit, the serine/threonine-protein kinase TAO1, and the #7 hit, Janus kinase 1 (JAK1), were also identified in this list. Future work will focus on validating the interactions of these candidate proteins with open form of tTG and determining whether they are linked to its cytotoxic effects.

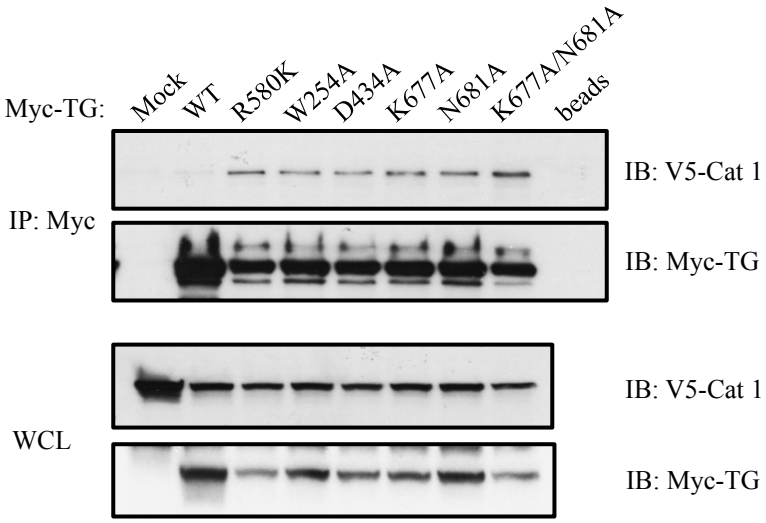
In another approach, we tested proteins that are known to be essential for cell viability or promote cell survival, and determined whether they preferentially associate with tTG R580K, compared with wild type tTG. We found that the protein GIT1/Cat-1 (G protein-coupled receptor kinase interacting ArfGAP 1/Cool associated tyrosine phosphorylated 1) strongly associated with tTG R580K and other open forms of tTG, but showed almost no affinity for wild type tTG (Figure 4.1A). Cat-1 is best known for its roles in regulating cell shape and cell migration, by functioning as a scaffold protein and bringing various proteins together through its different protein binding motifs (*31*). However, recent studies from our lab showed that Cat-1 expression is up-regulated in human cervical cancer samples, and that it contributes to their transformed characteristics (*32*). We found that knocking-down Cat-1 expression with siRNA in HeLa cells triggered a strong apoptotic response, similar to what we have observed when over-expressing tTG R580K in these cells (Figure 4.1B). Thus, an attractive working model is that the open form of tTG, by binding to and sequestering Cat-1, disrupts the normal signaling and cellular functions mediated by Cat-1 and therefore triggers cell death. We are currently in the process of determing the domain on Cat-1 that binds to tTG R580K, with the hope being that

Figure 4.1. Cat-1 binds to open forms of tTG.

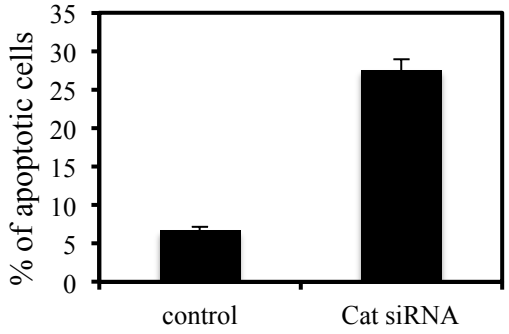
(A) Immunoprecipitations with a Myc antibody (IP: Myc) were performed on extracts from 293T cells transfected with V5-tagged Cat-1 and either Myc-tagged wild type tTG (WT) or with the indicated Myc-tagged mutant forms of tTG. The resulting immuno-complexes, as well as the whole cell extracts collected (WCL), were immunoblotted (IB) with Myc and V5 antibodies.

(B) Apoptotic assays were performed on HeLa cells that were transfected with control siRNA or a siRNA targeting Cat-1. Percentages of apoptotic cells under each condition are plotted. Data are represented as mean ± SEM.

A


Myc-TG:
Mock
WT
R580K
W254A
D434A
K677A
N681A
K677A/N681A
beads
IP: Myc
IB: V5-Cat 1
IB: Myc-TG
WCL
IB: V5-Cat 1
IB: Myc-TG

B


% of apoptotic cells
35
30
25
20
15
10
5
0
control
Cat siRNA

these findings will allow us to generate mutant forms of Cat-1 that no longer interact with tTG and then examine how they influence cell viability.

Closing Remarks

An increasing number of studies over the past decade has greatly expanded our understanding of the biochemical, structural, and functional activities of tTG, an enzyme that was mostly recognized for its crosslinking activity, but is now appreciated to be a multifunctional protein that plays important roles in cell differentiation, ECM stabilization, neurodegeneration and cancer progression. During the course of this thesis work, I have built upon these previous findings and identified a new role for tTG in promoting brain tumor progression, as well as uncovered the molecular mechanisms that regulate the transition of tTG between two functionally distinct states. Hopefully, these new insights will help us to better understand the function and regulation of tTG, and provide some new possibilities for targeting aberrant tTG functions in the treatment of different disease conditions.

REFERENCES

1. Li, B., Cerione, R. A., and Antonyak, M. (2011) Tissue transglutaminase and its role in human cancer progression, *Advances in enzymology and related areas of molecular biology 78*, 247-293.

2. Mehta, K., Kumar, A., and Kim, H. I. (2010) Transglutaminase 2: a multi-tasking protein in the complex circuitry of inflammation and cancer, *Biochemical pharmacology 80*, 1921-1929.

3. Li, B., Antonyak, M. A., Druso, J. E., Cheng, L., Nikitin, A. Y., and Cerione, R. A. (2010) EGF potentiated oncogenesis requires a tissue transglutaminase-dependent signaling pathway leading to Src activation, *Proceedings of the National Academy of Sciences of the United States of America 107*, 1408-1413.

4. Verma, A., and Mehta, K. (2007) Tissue transglutaminase-mediated chemoresistance in cancer cells, *Drug resistance updates : reviews and commentaries in antimicrobial and anticancer chemotherapy 10*, 144-151.

5. Boroughs, L. K., Antonyak, M. A., Johnson, J. L., and Cerione, R. A. (2011) A unique role for heat shock protein 70 and its binding partner tissue transglutaminase in cancer cell migration, *The Journal of biological chemistry 286*, 37094-37107.

6. Raizer, J. J. (2005) HER1/EGFR tyrosine kinase inhibitors for the treatment of glioblastoma multiforme, *Journal of neuro-oncology 74*, 77-86.

7. Shinojima, N., Tada, K., Shiraishi, S., Kamiryo, T., Kochi, M., Nakamura, H., Makino, K., Saya, H., Hirano, H., Kuratsu, J., Oka, K., Ishimaru, Y., and Ushio, Y. (2003) Prognostic value of epidermal growth factor receptor in patients with glioblastoma multiforme, *Cancer research 63*, 6962-6970.

8. Huang, P. H., Xu, A. M., and White, F. M. (2009) Oncogenic EGFR signaling networks in glioma, *Science signaling 2*, re6.

9. Levkowitz, G., Waterman, H., Zamir, E., Kam, Z., Oved, S., Langdon, W. Y., Beguinot, L., Geiger, B., and Yarden, Y. (1998) c-Cbl/Sli-1 regulates endocytic sorting and ubiquitination of the epidermal growth factor receptor, *Genes & development 12*, 3663-3674.

10. Waterman, H., Levkowitz, G., Alroy, I., and Yarden, Y. (1999) The RING finger of c-Cbl mediates desensitization of the epidermal growth factor receptor, *The Journal of biological chemistry 274*, 22151-22154.

11. Li, J. J., Liu, D. P., Liu, G. T., and Xie, D. (2009) EphrinA5 acts as a tumor suppressor in glioma by negative regulation of epidermal growth factor receptor, *Oncogene 28*, 1759-1768.

12. Vivanco, I., Rohle, D., Versele, M., Iwanami, A., Kuga, D., Oldrini, B., Tanaka, K., Dang, J., Kubek, S., Palaskas, N., Hsueh, T., Evans, M., Mulholland, D., Wolle, D., Rajasekaran, S., Rajasekaran, A., Liau, L. M., Cloughesy, T. F., Dikic, I., Brennan, C., Wu, H., Mischel, P. S., Perera, T., and Mellinghoff, I. K. (2010) The phosphatase and tensin homolog regulates epidermal growth factor receptor (EGFR) inhibitor response by targeting EGFR for degradation, *Proceedings of the National Academy of Sciences of the United States of America 107*, 6459-6464.

13. Feng, Q., Baird, D., Peng, X., Wang, J., Ly, T., Guan, J. L., and Cerione, R. A. (2006) Cool-1 functions as an essential regulatory node for EGF receptor- and Src-mediated cell growth, *Nature cell biology 8*, 945-956.

14. Zhang, R., Tremblay, T. L., McDermid, A., Thibault, P., and Stanimirovic, D. (2003) Identification of differentially expressed proteins in human glioblastoma cell lines and tumors, *Glia 42*, 194-208.

15. Deshaies, R. J., and Joazeiro, C. A. (2009) RING domain E3 ubiquitin ligases, *Annu Rev Biochem 78*, 399-434.

16. Miyake, S., Mullane-Robinson, K. P., Lill, N. L., Douillard, P., and Band, H. (1999) Cbl-mediated negative regulation of platelet-derived growth factor receptor-dependent cell proliferation. A critical role for Cbl tyrosine kinase-binding domain, *The Journal of biological chemistry 274*, 16619-16628.

17. Abella, J. V., Peschard, P., Naujokas, M. A., Lin, T., Saucier, C., Urbe, S., and Park, M. (2005) Met/Hepatocyte growth factor receptor ubiquitination suppresses transformation and is required for Hrs phosphorylation, *Molecular and cellular biology 25*, 9632-9645.

18. Kaabeche, K., Lemonnier, J., Le Mee, S., Caverzasio, J., and Marie, P. J. (2004) Cbl-mediated degradation of Lyn and Fyn induced by constitutive fibroblast growth factor receptor-2 activation supports osteoblast differentiation, *The Journal of biological chemistry 279*, 36259-36267.

19. Stettner, M. R., Wang, W., Nabors, L. B., Bharara, S., Flynn, D. C., Grammer, J. R., Gillespie, G. Y., and Gladson, C. L. (2005) Lyn kinase activity is the predominant cellular SRC kinase activity in glioblastoma tumor cells, *Cancer research 65*, 5535-5543.

20. Betsholtz, C., Nister, M., Rorsman, F., Heldin, C. H., and Westermark, B. (1989) Structural and functional aspects of platelet-derived growth factor and its role in the pathogenesis of glioblastoma, *Molecular and chemical neuropathology / sponsored by the International Society for Neurochemistry and the World Federation of Neurology and research groups on neurochemistry and cerebrospinal fluid 10*, 27-36.

21. Moriyama, T., Kataoka, H., Koono, M., and Wakisaka, S. (1999) Expression of hepatocyte growth factor/scatter factor and its receptor c-Met in brain tumors: evidence for a role in progression of astrocytic tumors (Review), *International journal of molecular medicine 3*, 531-536.

22. Nakaoka, H., Perez, D. M., Baek, K. J., Das, T., Husain, A., Misono, K., Im, M. J., and Graham, R. M. (1994) Gh: a GTP-binding protein with transglutaminase activity and receptor signaling function, *Science 264*, 1593-1596.

23. Lee, M. Y., Chung, S., Bang, H. W., Baek, K. J., and Uhm, D. (1997) Modulation of large conductance Ca2+-activated K+ channel by Galphah (transglutaminase II) in the vascular smooth muscle cell, *Pflugers Arch 433*, 671-673.

24. Liu, S., Cerione, R. A., and Clardy, J. (2002) Structural basis for the guanine nucleotide-binding activity of tissue transglutaminase and its regulation of transamidation activity, *Proceedings of the National Academy of Sciences of the United States of America 99*, 2743-2747.

25. Pinkas, D. M., Strop, P., Brunger, A. T., and Khosla, C. (2007) Transglutaminase 2 undergoes a large conformational change upon activation, *PLoS Biol 5*, e327.

26. Grosso, H., and Mouradian, M. M. (2012) Transglutaminase 2: biology, relevance to neurodegenerative diseases and therapeutic implications, *Pharmacology & therapeutics 133*, 392-410.

27. Chishti, A. H., Kim, A. C., Marfatia, S. M., Lutchman, M., Hanspal, M., Jindal, H., Liu, S. C., Low, P. S., Rouleau, G. A., Mohandas, N., Chasis, J. A., Conboy, J. G., Gascard, P., Takakuwa, Y., Huang, S. C., Benz, E. J., Jr., Bretscher, A., Fehon, R. G., Gusella, J. F., Ramesh, V., Solomon, F., Marchesi, V. T., Tsukita, S., Tsukita, S., Hoover, K. B., and et al. (1998) The FERM domain: a unique module involved in the linkage of cytoplasmic proteins to the membrane, *Trends in biochemical sciences 23*, 281-282.

28. Frame, M. C., Patel, H., Serrels, B., Lietha, D., and Eck, M. J. (2010) The FERM domain: organizing the structure and function of FAK, *Nature reviews. Molecular cell biology 11*, 802-814.

29. Goldie, S. J., Mulder, K. W., Tan, D. W., Lyons, S. K., Sims, A. H., and Watt, F. M. (2012) FRMD4A upregulation in human squamous cell carcinoma promotes tumor growth and metastasis and is associated with poor prognosis, *Cancer research 72*, 3424-3436.

30. Ikenouchi, J., and Umeda, M. (2010) FRMD4A regulates epithelial polarity by connecting Arf6 activation with the PAR complex, *Proceedings of the National Academy of Sciences of the United States of America 107*, 748-753.

31. Hoefen, R. J., and Berk, B. C. (2006) The multifunctional GIT family of proteins, *Journal of cell science 119*, 1469-1475.

32. Yoo, S. M., Antonyak, M. A., and Cerione, R. A. (2012) The adaptor protein and Arf GTPase-activating protein Cat-1/Git-1 is required for cellular transformation, *The Journal of biological chemistry 287*, 31462-31470.